Energiekonservierung über Organohalid-Respiration in *Sulfurospirillum multivorans*

Dissertation

zur Erlangung des akademischen Grades

„doctor rerum naturalium“ (Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät

der Friedrich-Schiller-Universität Jena

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Geboren am 17.03.1983 in Bayreuth

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Tag der öffentlichen Disputation: 18.01.2018

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1. Einleitung

1.1 Topologie anaerober Atmungsketten

Die Atmung ist für den aeroben und anaeroben Energiestoffwechsel vieler prokaryotischer Organismen von grundlegender Bedeutung. Grundsätzlich wird die freie Energie einer durch eine membrangebundene Elektronentransportkette katalysierten Redoxreaktion verwendet um die Translokation von Protonen über eine Kopplungsmembran anzutreiben und dadurch einen elektrochemischen Ionengradienten (üblicherweise Protonengradienten), auch Protonenmotorische Kraft (PMK) genannt, zu erzeugen. Anschließend wird die PMK zur Synthese von ATP durch die ATP-Synthase genutzt. Die PMK setzt sich aus zwei Größen, dem elektrischen Membranpotential $\Delta\Psi$ (in mV) und dem Protonendiffusionspotential (ΔpH), welches proportional zur pH-Differenz zwischen Außen- und Innenseite der Membran ist, zusammen. Die Protonenmotorische Kraft kann durch verschiedene Mechanismen wie Protonenpumpen, Chinon/ Chinol-Kreisläufe oder durch eine Redoxschleife aufgebaut werden. Redoxschleifen finden sich häufig in anaeroben Atmungssystemen, bei denen der ΔG-Wert der Redoxreaktion kleiner ist als bei der aeroben Atmung und wo daher kürzere Atmungsketten und weniger Ionenpumpenzyme zu finden sind (Simon *et al.*, 2008). Solche Redoxschleifen bestehen häufig aus zwei Chinon-reaktiven Enzymen, die einen modularen Aufbau aufweisen, sowie einem Chinon, das als Elektronenüberträger zwischen den Enzymen fungiert (Simon *et al.*, 2008). Hier wird die PMK durch eine unterschiedliche Lokalisation sowohl der Reaktionszentren als auch der Chinon-/ Chinol-Bindestellen der an der Redoxschleife beteiligten Enzyme erzeugt. Gut untersuchte anaerobe Atmungssysteme, in denen die PMK durch Redoxschleifen erzeugt wird, sind die Formiat-abhängige Fumarat- und Nitrat-Atmung von *Wolinella succinogenes* (Abbildung 1-1). Bei der Formiat-Dehydrogenase (FdhABC) von *W. succinogenes* sind das Reaktionszentrum für die Oxidation des Elektronendonors und die Chinon-/Chinol-Bindestelle an entgegengesetzten Seiten der Zytoplasmamembran lokalisiert (Abbildung 1-1). Enzyme, die eine solche Lokalisation der Reaktionszentren aufweisen, sind an dem Aufbau der PMK beteiligt; pro in der Redoxreaktion freigesetztem bzw. verbrauchtem Elektron wird ein Proton über die Membran transloziert (Simon *et al.*, 2008). Formiat wird durch die periplasmatisch lokalisierte Formiat-Dehydrogenase (Untereinheit FdhA) oxidiert wobei Protonen in das Periplasma freigesetzt werden (Jankielewicz *et al.*, 2004). Die Weiterleitung der bei der Oxidation von Formiat generierten Elektronen erfolgt im Enzym über die Eisen-Schwefel-Zentren der Untereinheiten FdhA und FdhB auf ein Low-Potential-Cytochrom *b* der membranständigen Untereinheit FdhC (E_{SHE} = -200 mV; Kröger &

Innerhofer, 1976). Nachfolgend werden die Elektronen auf Menachinon (E_{SHE} = -74 mV, bei pH 7,0; Thauer *et al.*, 1977) übertragen, wobei Menachinon unter Aufnahme von Protonen aus dem Zytoplasma zu Menachinol reduziert wird (Abbildung 1-1) (Simon *et al.*, 2008). Ist Fumarat der Elektronenakzeptor, wird das gebildete Menachinol an der Menachinolbindestelle der Fumarat-Reduktase-Untereinheit FrdC unter Freisetzung von Protonen oxidiert und die Elektronen auf ein High-Potential-Cytochrom *b* übertragen (E_{SHE} = -20 mV; Unden *et al.,* 1980; Kröger *et al.*, 2002). Während die katalytische, für die Reduktion von Fumarat zuständige Untereinheit der Fumarat-Reduktase (FrdA) cytoplasmatisch orientiert ist, ist die Chinon-/Chinol-Bindestelle auf der periplamatischen Seite der Zytoplasmamembran lokalisiert (Abbildung 1-1A) (Lancaster *et al.*, 1999). Bei der Oxidation des Menachinols durch Fumarat sollte es sich daher um einen elektrogenen, PMK generierenden Prozess handeln. Jedoch konnte experimentell gezeigt werden, dass die Fumarat-Reduktion durch Menachinol nicht mit einer Protonentranslokation über die Membran gekoppelt ist (Biel *et al.*, 2002). Die Lösung dieses Widerspruchs stellt der experimentell nachgewiesene „E-Weg"-Mechanismus dar (Lancaster *et al.*, 2006). Nach diesem Mechanismus werden die während der Menachinol-Oxidation frei werdenden Protonen in das Periplasma entlassen, gleichzeitig findet aber ein Kotransport von zwei Protonen über die Membran in das Zytoplasma statt (Abbildung 1-1A). Wie bei der Fumarat-Atmung wird auch bei der Nitrat-Atmung von *W. succinogenes* die PMK allein durch die Reduktion von Menachion durch Formiat generiert, während es sich bei der Nitrat-Reduktion um einen elektroneutralen Prozess handelt. Terminale Reduktase der Nitrat-Respiration von *W. succinogenes* ist die periplasmatische Nitrat-Reduktase NapA. Die Oxidation des Menachinols sowie die Weiterleitung der Elektronen an NapA erfolgt in der Nitrat-Respiration wahrscheinlich durch die putative Chinol-Dehydrogenase NapGH (Abbildung 1-1B). NapH ist ein membranintegrales Protein mit zwei [4Fe-4S] Clustern und enthält vermutlich eine Chinol-Bindestelle von der Elektronen an das periplasmatisch lokalisierte Eisen-Schwefel-Protein NapG übertragen und anschließend über das periplasmatische di-Häm Cytochrom *c* (NapB) an NapA weitergeleitet werden (Abbildung 1-1B) (Kern *et al.*, 2007; Kern & Simon, 2008).

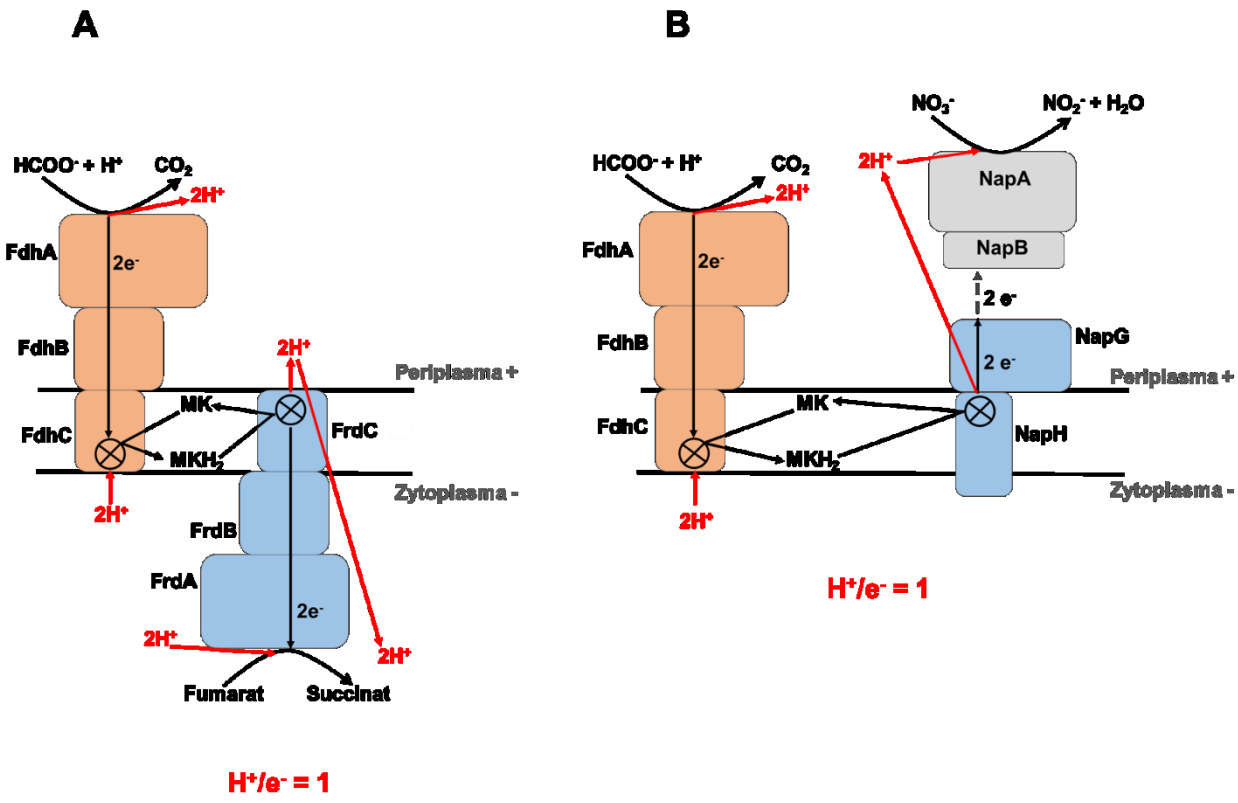

Abbildung 1-1: Modell der Fumarat- und Nitrat-Atmungskette von *Wolinella succinogenes* mit Formiat als Elektronendonor. Fdh: Formiat-Dehydrogenase (FdhA, B, C), integriert in die Zytoplasmamembran durch die di-Häm Cytochrom *b* Untereinheit (FdhC); MK/MKH_2: Menachinon/Menachinol. **A: Fumarat-Atmung.** Frd: Fumarat-Reduktase (FrdA, B, C), integriert in die Zytoplamamembran durch die di-Häm Cytochrom *b* Untereinheit (FrdC). **B: Nitrat-Atmung**. NapA: Nitrate-Reduktase, katalytisch aktive Untereinheit; NapB: di-Häm *c*-Typ Cytochrom; NapGH: putative Chinol-Dehydrogenase. ■: Chinon-/Chinol-Bindestelle.

1.2 Organohalide in der Umwelt

Organohalide (halogenierte Kohlenwasserstoffe) sind in der Natur ubiquitär vorkommende Verbindungen. Entgegen den früheren Annahmen, dass Organohalide bis auf wenige Ausnahmen (Gribble, 2003) rein anthropogenen Ursprungs sind, ist heute bekannt, dass diese Verbindungen ebenfalls durch natürliche Prozesse gebildet werden. Waren 1968 gerade einmal 30 natürliche Organohalide bekannt, sind es heute über 5000 sowohl biotischen als auch abiotischen Ursprungs (Gribble, 2012). So vielfältig die biotisch gebildeten Organohalide sind, so unterschiedlich sind auch die Organismen, die diese strukturell und biologisch außergewöhnlichen Verbindungen synthetisieren (Gribble, 1998). Neben marinen Organismen, wie Rot- und Blaualgen (z.B. *Asparagopsis taxiformis*, *Lyngybya majuscula*), Schwämmen, Seescheiden (*Ascidiaceae*) oder

Weichkorallen (*Alyconacea*), gibt es auch terrestrische Pflanzen, Pilze, Bakterien und Insekten, die halogenierte Verbindungen synthetisieren. So gehören zu den zuerst entdeckten natürlichen Organohaliden die von Pilzen synthetisierten chlorhaltigen, antibiotisch wirksamen Substanzen Griseofulvin und Chlorampenicol (Gribble, 1998; Gribble 2003). Neben den genannten biotischen Quellen sind auch verschiedene abiotische Prozesse für die Bildung von halogenierten organischen Verbindungen verantwortlich. So stellen Vulkanausbrüche sowie Wald- bzw. Buschbrände natürliche abiotische Quellen für Organohalide dar.
Große Mengen an Organohaliden werden für verschiedene Verwendungszwecke industriell hergestellt. Sie finden Anwendung als Lösungsmittel (z.B. Tetrachlorethen oder Trichlorethen), sind Bestandteil von Extraktionsmitteln, Schmier- und Farbstoffen und dienen als Ausgangsstoffe für die Herstellung von Pestiziden (z.B. γ-Hexachlorcyclohexan „Lindan“). Aufgrund ihrer intensiven Nutzung in der Industrie und Landwirtschaft sowie ihrem teilweise unsachgemäßen Gebrauch sind Organohalide heute in relativ hohen Konzentrationen (z.B. Konzentrationen von bis zu mehrere 100 Mikrogramm pro Liter Grundwasser für Tetrachlorethen; Fetzner, 1998) in der Umwelt vorhanden, wo sie auf Grund ihres chemischen Charakters ein zum Teil erhebliches Umwelt- und Gesundheitsrisiko darstellen.

1.3 Organohalid-Respiration

Das Vorkommen natürlich produzierter halogenierter Kohlenwasserstoffe könnte zur Entwicklung verschiedener biochemischer Strategien beigetragen haben, die ein Aufbrechen der chemisch stabilen Kohlenstoff-Halogen-Bindungen in Organohaliden ermöglichten. Dies ist vor allem für die Sauerstoff-unabhängigen Dehalogenierungsprozesse, die sich wahrscheinlich in der ursprünglich sauerstofffreien Atmosphäre auf der Erde entwickelt haben, von besonderer Bedeutung (Atashgahi *et al.*, 2016). So entstanden im Laufe der Evolution in allen phylogenetischen Reichen (Bacteria, Archaea, Eukarya) zur Dehalogenierung befähigte Organismen.

Biotischer Abbau halogenierter Verbindungen

Der biotische Abbau halogenierter organischer Verbindungen erfolgt durch diverse mikrobielle Systeme, wobei die beteiligten Bakterien abhängig von den Umweltbedingungen und der Art der zu dehalogenierenden Substanzen sich dabei unterschiedlicher Abbauwege bedienen. So kann der Abbau bzw. die Dehalogenierung von Organohaliden über Fermentation wie zum Beispiel für *Dehalobacterium formicoaceticum* beschrieben, das Dichlormethan zu Acetat und Formiat

umsetzt (Mägli *et al.*, 1996), sowie auch über oxidative oder reduktive Dehalogenierung erfolgen. Niederchlorierte Verbindungen wie cis-1,2-Dichlorethen oder Vinylchlorid werden recht effizient unter aeroben Bedingungen (oxidative Dehalogenierung) mikrobiell abgebaut. Dieser Abbau erfolgt allerdings cometabolisch meist durch Mono- und Dioxygenasen ohne Energiegewinn für die Bakterien (Fetzner, 1998). Im Gegensatz dazu kann die Dehalogenierung mehrfach halogenierter Kohlenwasserstoffe wie zum Beispiel Tetrachlorethen (PCE), polychlorierte Biphenyle (PCB) oder Hexachlorbenzole (Fetzner, 1998) nur unter reduktiven, das bedeutet anaeroben Bedingungen, erfolgen. Heute ist eine Vielzahl an anaeroben Bakterien bekannt, die solche mehrfach halogenierten Verbindungen reduktiv zu dehalogenieren vermögen. Ist dabei der Prozess der reduktiven Dehalogenierung an die Energiegewinnung, also an die Synthese von ATP mittels Elektronentransportphosphorylierung gekoppelt, spricht man von Organohalid-Respiration (Leys *et al.*, 2013). Bakterien mit der Befähigung Organohalid-Respiration zu betreiben, werden als Organohalid-atmenden Bakterien, kurz OHRB (organohalide-respiring bacteria; Atashgahi *et al.*, 2016) bezeichnet.

Reduktive Dehalogenierung von Tetrachlorethen (PCE)

Auf Grund ihres sehr positiven Redoxpotentials stellen PCE und seine Dechlorierungsprodukte ideale Elektronenakzeptoren für die Organohalidrespiration dar. In anaeroben mikrobiellen Gemeinschaften wird PCE schrittweise über Trichlorethen (TCE), cis-Dichlorethen (cDCE) und Vinylchlorid (VC) zu Ethen reduktiv dechloriert (Abbildung 1-2). Die meisten Bakterien, wie zum Beispiel *Sulfurospirillum multivorans* (Goris & Diekert, 2016) oder *Desulfitobacterium hafniense* Y51 (Futagami & Furukama, 2016), die PCE über Organohalid-Respiration zu dechlorieren vermögen, bauen PCE nur unvollständig ab. Eine komplette Dechlorierung von PCE zu Ethen unter anaeroben Bedingungen konnte erstmals für *Dehalococcoides mccartyi* Stamm 195 nachgewiesen werden. Dabei handelt es sich für den Abbau von PCE zu Vinylchlorid um einen metabolischen, also an die Energiegewinnung gekoppelten Prozess, während die Umsetzung von Vinylchlorid zu Ethen co-metabolisch ohne Energiekonservierung erfolgt (Mayamó-Gatell *et al.*, 1997). Eine Kopplung aller Stufen der Dechlorierung von PCE an die Energiekonservierung konnte für *Dehalococcoides* sp. BTF08 gezeigt werden (Cichocka *et al.*, 2010).

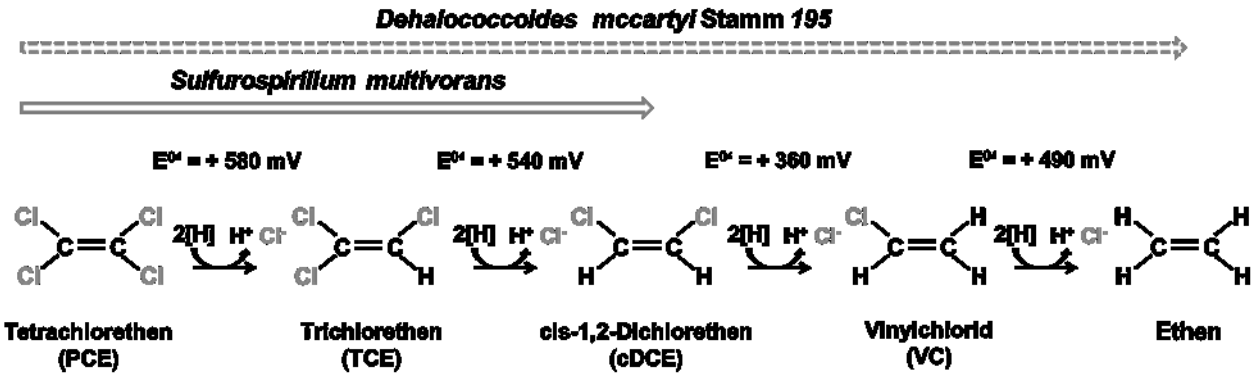

Abbildung 1-2: Vollständige Dechlorierung von Tetrachlorethen zu Ethen durch anaerobe reduktive Dechlorierung. Während PCE von *Sulfurospirillum multivorans* nur bis zur Stufe von cDCE abgebaut wird, baut *Dehalococcoides mccartyi* Stamm 195 PCE vollständig zu Ethen ab. Die Angabe der Redoxpotentiale basiert auf Vogel *et al.*, 1987.

Organohalid-Respiration in gram-negativen und gram-positiven Bakterien

Die heute bekannten OHRB lassen sich in vier phylogenetisch verschiedene Klassen einordnen: δ-Proteobakterien, ε-Proteobakterien, Chloroflexi (Dehalococcoidetes) und gram-positive Bakterien mit niedrigem G+C-Gehalt (Firmicutes) (Abbildung 1-3).

Erstmals wurde die Organohalid-Respiration für das zur Klasse der Gammaproteobakterien gehörende Bakterium *Desulfomonile tiedjei* für die Deaholgenierung von 3-Chlorobenzoat beschrieben (de Weerd & Sulfita, 1990; Mohn & Tiedjei, 1990). Weitere zur reduktiven Dehalogenierung befähigte Vertreter dieser Klasse sind *Desulfuromonas michiganensis* (Sung *et al.*, 2003) und *Anaeromyxobacter dehalogenans* (He *et al.*, 2002). Zu den reduktive Dehalogenierung-betreibenden Epsilonproteobakterien gehören Arten der Gattung *Sulfurospirillum* wie *S. multivorans* (Scholz-Muramatsu *et al.*, 1995) und *S. halorespirans* (Luijten *et al.*, 2003). Die zur phylogenetischen Gruppe der Chloroflexi gehörende Gattung Dehalococcoides umfasst ausschließlich reduktiv dehalogenierende Stämme wie *Dehalococcoides mccartyi* 195 oder *Dehalococcoides mccartyi* CBDB1. Auch in der phylogenetischen Gruppe der gram-positiven Bakterien mit niedrigem G+C-Gehalt (Firmicutes) finden sich Vertreter, die in der Lage sind, reduktive Dehalogenierung zu betreiben (Atashgahi *et al.*, 2016). Dazu gehören Arten der Gattung *Desulfitobacterium* wie zum Beispiel *D. hafniense* Stamm PCE-S (Granzow, 1998) und Stamm Y51 (Suyama *et al.*, 2001) oder *Dehalobacter restrictus* PER-K23 (Holliger *et al.*, 1998).

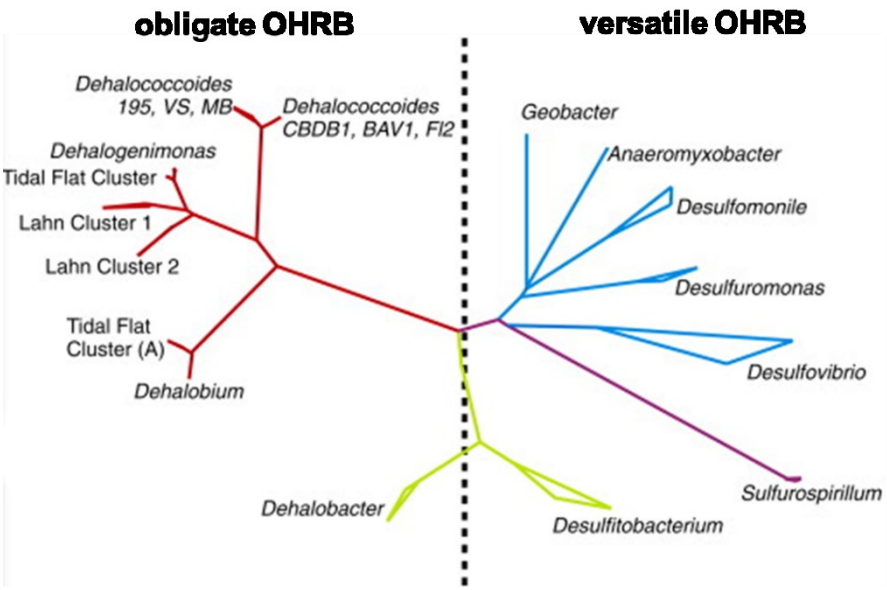

Abbildung 1-3: Phylogenetischer Stammbaum der OHRB, basierend auf 16S rRNA Sequenzen. Farbcode: Chloroflexi (rot), δ-Proteobakterien (blau), ε-Proteobakterien (violett), Firmicutes (grün). Verändert nach Maposa *et al.*, 2010.

Innerhalb der OHRB finden sich sowohl Arten, die bei ihrer Energiegewinnung strikt an die Organohalid-Respiration gebunden sind (obligate OHRB) als auch Bakterien, die einen versatilen Metabolismus im Hinblick auf die Energiegewinnung aufweisen (fakultative OHRB) (Atashgahi *et al.*, 2016) (Abbildung 1-3). Die fakultativen OHRB können neben Organohaliden meist ein breites Spektrum an alternativen Elektronenakzeptoren in ihrem respiratorischen Stoffwechsel verwenden oder auch fermentative Prozesse zur Energiegewinnung nutzen. Zu den fakultativen OHRB gehören zum Beispiel Arten der Gattungen *Geobacter*, *Desulfomonile* (Gammaproteobakterien), *Sulfurospirillum.* (Epsilonroteobakterium) oder auch *Desulfitobacterium.* (Firmicutes). Zu den obligaten OHRB, deren Metabolismus strikt an die Nutzung von Organohaliden als Elektronenakzeptoren gebunden ist, gehören Arten wie *Dehalococcoides spp.* oder *Dehalobacter spp.* (Firmicutes) (Atashgahi *et al.*, 2016). Die Unterschiede im Metabolismus von obligaten und fakultativen OHRB spiegeln sich auch im Spektrum der Elektronendonoren, die die verschiedenen Organismen nutzen können, wieder. Während die obligaten OHRB nahezu ausschließlich Wasserstoff als Elektronendonor nutzen können, verwenden die fakultativen OHRB sowohl organische Verbindungen, wie zum Beispiel Pyruvat oder Lactat, als auch Formiat und Wasserstoff als Elektronendonoren (Mayer-Blackwell *et al.*, 2016).

Charakterisierung reduktiver Dehalogenasen

Die Schlüsselenzyme der Organohalid-Respiration sind die reduktiven Dehalogenasen (RDasen) (Atashgahi *et al.*, 2016). Die meisten der beschriebenen Dehalogenasen zeigen dabei eine Präferenz zur Dehalogenierung von entweder aliphatischen oder aromatischen halogenierten Kohlenwasserstoffen. Beispiele für Enzyme, die aliphatische Organohalide dehalogenieren, sind die PCE-RDasen (PceAs) aus *S. multivorans* (Neumann *et al.*, 1996), *Desulfitobacterium hafniense* PCE-S (Miller *et al.*, 1998) oder *Dehalobacter restrictus* (Maillard *et al.*, 2003). Aromatische Organohalide werden über Enzyme wie zum Beispiel der ortho-Chlorophenol-RDase (CprA) von *Desulfitobacterium dehalogenans* (van de Pas *et al.*, 1999) dehalogeniert.

Alle bisher gereinigten und charakterisierten Dehalogenasen, mit Ausnahme der 3-Chlorobenzoat-reduktiven Dehalogenase von *Desulfomonile tiedjei*, sind Eisen-Schwefel-Proteine, die einen Corrinoid-Kofaktor enthalten (Schubert & Diekert, 2016). Als Monomere vorliegend weisen sie eine molare Masse zwischen 46 und 65 kDa auf. Allerdings ist nicht bekannt, ob die RDasen unter natürlichen Bedingungen als Monomere oder Oligomere vorkommen. Für PceA von *S. multivorans* konnte durch Strukturanalyse gezeigt werden, dass dieses Enzym unter natürlichen Bedingungen mit sehr großer Wahrscheinlichkeit als Homodimer vorliegt (Bommer *et al.*, 2014: Schubert & Diekert et al., 2016). Als bisher einzige beschriebene Dehalogenase enthält die 3-Chlorobenzoat-reduktive Dehalogenase von *D. tiedjei* anstelle des Corrinoids ein Häm als Kofaktor und liegt als heterodimerer Komplex vor (Ni *et al.*, 1995; Schubert & Diekert, 2016). Neben dem Corrinoid enthalten alle bisher beschriebenen reduktiven Dehalogenasen mit Ausnahme der reduktiven Dehalogenase von *D. tiedjei* (Ni *et al.*, 1995) zwei Eisen-Schwefel-Cluster (Fe-S) als Kofaktoren (Schubert & Diekert, 2016). Alle Dehalogenasen weisen zwei konservierte Bindemotive für Fe-S Cluster am C-terminalen Ende auf, die zwei [4Fe-4S] Cluster oder alternativ einen [4Fe-4S] und einen [3Fe-4S] Cluster binden (Schubert & Diekert, 2016). Die zweite Variante wurde bislang nur für die ortho-Chlorophenol-reduktive Dehalogenase (CprA) von *D. dehalogenans* beschrieben (van de Pas *et al.*, 1999).

Die physiologisch aktive, maturierte Form der Dehalogenasen ist im Exoplasma der Bakterienzellen bzw. Periplasma bei gram-negativen Bakterien lokalisiert und liegt mit der Zytoplasmamembran assoziiert vor (Nijenhuis & Zinder, 2005; John *et al.*, 2006; Reinhold *et al.*, 2012). Alle bisher bekannten reduktiven Dehalogenasen vom PceA- und CprA-Typ werden als cytoplasmatische Vorläuferproteine produziert, die am N-Terminus ein Signalpeptid mit der charakteristischen Tat-Konsensus-Sequenz „RRxFxK“ tragen (Smidt & de Vos, 2004; Schubert & Diekert, 2016). Dieses Signalpeptid ist für die Erkennung des Vorläufers durch das Tat-System (Tat: twin arginine translocation), welches dem Transport Kofaktor-haltiger Enzyme durch die

Zytoplasmamembran dient, notwendig (Palmer & Berks, 2012). Die Assoziation der respiratorischen RDasen mit der Zytoplasmamembran ist wahrscheinlich auf eine Interaktion der Dahalogenasen mit dem putativen membranintegralen B-Protein (PceB bzw. CrpB) zurückzuführen (Neumann *et al.*, 1998).

Die Anzahl der für reduktive Dehalogenasen kodierenden Gene (*rdhA*) in den Genomen der OHRB kann zwischen weniger als 10 in den fakultativen OHRB, wie zum Beispiel *Sulfurospirillum* spp. oder *Desulfitobacterium* spp., und mehr als 30 in den obligaten OHRB wie *Dehalococcoides* spp. variieren (Hug *et al.*, 2013; Kruse *et al.*, 2016).
Die Gene der reduktiven Dehalogenasen sind in der Regel in einem Operon organisiert, dass neben dem *rdhA* Gen, das die katalytischen Untereinheiten der Dehalogenasen kodiert, noch das kleines Gen *rdhB* enthält (Neumann *et al.*, 1998; Futagami *et al.*, 2008; Kruse *et al.*, 2016). Das Gen *rdhB* kodiert für ein hydrophobes Protein, das wahrscheinlich als Membrananker für die reduktiven Dehalogenasen fungiert (Neumann *et al.*, 1998; Futagami *et al.*, 2008; Kruse *et al.*, 2016). Diese minimalen *rdhAB*-Operons werden regelmäßig von zusätzlichen Genen, wobei die Anzahl und Art der Gene variiert, begleitet. Die Funktion der meisten dieser Gene ist bis heute unbekannt (Kruse *et al*., 2016). Einige der Gene kodieren für Proteine, die an der Regulation der Expression der *rdhAB*-Gene beteiligt sind (Wagner *et al.*, 2013; Gábor *et al.*, 2008; Pop *et al.*, 2004), während andere als Chaperone fungieren, die wahrscheinlich bei der Faltung der Dehalogenasen eine Rolle spielen (Maillard *et al.*, 2011; Mac Nelly *et al.*, 2014; Morita *et al.*, 2009).

1.4 *Sulfurospirillum multivorans*

Die einzigen bis heute beschrieben ε-Proteobakterien, die Organohalid-Respiration betreiben, gehören der Gattung *Sulfurospirillum* an. Bei den meisten *Sulfurospirillum* Arten handelt es sich um physiologisch versatile, oft mikroaerophile Organismen, die mit einer Vielzahl an unterschiedlichen Elektronendonoren und –akzeptoren wachsen können. Während nur wenige Arten Organohalide als Substrate nutzen können, wachsen viele von ihnen mit anderen toxischen Substanzen wie zum Beispiel Arsenat oder Selenat. *Sulfurospirillum* Arten sind weltweit in Sedimenten, im Grundwasser oder im Boden sowie an Standorten, die eine Kontamination mit Organohaliden, Arsenat oder Selenat aufweisen, zu finden (Goris & Diekert, 2016).

S. multivorans wurde aus dem Belebtschlamm einer Kläranlage in Stuttgart-Büsnau mit Pyruvat als Elektronendonor und PCE als Elektronenakzeptor isoliert (Scholz-Muramatsu *et al.*, 1995).

Alternativ kann *S. multivorans* auch Wasserstoff oder Formiat als Elektronendonor für die reduktive Dehalogenierung nutzen. Durch die Verwendung von Wasserstoff bzw. Formiat als einziger Quelle für Reduktionsäquivalente konnte eine Energiegewinnung über Substratstufenphosphorylierung, die bei Verwendung von komplexeren organischen Verbindungen wie Pyruvat als Elektronendonor möglich wäre, ausgeschlossen werden. Somit stellt die von *S. multivorans* betriebene reduktive Dehalogenierung die terminale Reaktion einer Elektronentransportkette dar, die an die Energiegewinnung über Elektronentransport-phosphorylierung gekoppelt ist. Neben *S. multivorans* ist *S. halorespirans* die einzige *Sulfurospirillum* Art, für die die Energiegewinnung durch Organohalid-Respiration bislang eindeutig nachgewiesen werden konnte (Luijten *et al.*, 2003; Goris & Diekert, 2016).
Wie alle *Sulfurospirillum* Spezies kann *S. multivorans* sowohl organotroph als auch lithotroph wachsen, wobei im letzteren Fall Acetat als Kohlenstoffquelle benötigt wird (Goris & Diekert, 2016). Dabei kann der Organismus als fakultativer OHRB ein breites Substratspektrum zur Energiegewinnung nutzen. Neben den bereits erwähnten Substraten Pyruvat, Wasserstoff und Formiat, kann *S. multivorans* auch Natriumdisulfid, Lactat und Glycerol als Elektronendonoren verwenden. Als Elektronenakzeptoren können neben PCE und TCE Fumarat, Nitrat oder auch Sauerstoff (1 – 5% in der Gasphase) dienen (Scholz-Muramatsu, 1995; Goris & Diekert, 2016). Daneben ist *S. multivorans* bei der Energiegewinnung nicht allein auf respiratorische Prozesse angewiesen, sondern kann ebenfalls durch die Fermentation von Pyruvat bzw. Fumarat wachsen (Goris & Diekert, 2016).

Fumarat- und Nitrat-Respiration in *S. multivorans*

Neben Organohaliden kann *S. multivorans* auch Fumarat und Nitrat als Elektronenakzeptoren in einer anaeroben Atmungskette verwenden. Im Gegensatz zum Epsilonproteobakterium *Wolinella succinogenes* (siehe Kapitel 1.1) ist die Fumarat- und Nitrat-Respiration in *S. multivorans* bislang nicht sehr gut untersucht. Aufgrund der Verwandtschaft von *S. multivorans* und *W. succinogenes* kann von einem ähnlichen Aufbau sowie von einer vergleichbaren Funktionsweise dieser beiden anaeroben Atmungsketten in beiden Organismen ausgegangen werden. Die periplasmatisch orientierte Formiat-Dehydrogenase von *S. multivorans* besteht analog zum Enzym von *W. succinogenes* aus drei Untereinheiten - der katalytischen Molybdän- oder Wolfram-haltigen Untereinheit FdhA, der Eisen-Schwefel-Cluster-Untereinheit FdhB und der membranintegralen Cytochrom-*b*-Untereinheit FdhC (Schmitz & Diekert, 2004). Die Untereinheit FdhC besitzt ein putatives Chinonbindemotiv und vermittelt mit sehr großer Wahrscheinlichkeit analog zur Formiat-Dehydrogenase von *W. succinogenes* die Reduktion von Menachinon. Terminale Reduktase der

Fumarat-Atmung ist die Fumarat-Reduktase, die wahrscheinlich analog zur Fumarat-Reduktase von *W. succinogenes* cytoplasmatisch orientiert ist (Miller *et al.*, 1996). Terminale Reduktase der Nitrat-Respiration ist die periplasmatische Nitrat-Reduktase NapA. Wie in *W. succinogenes* ist der Proteinkomplex NapGH wahrscheinlich an der Oxidation von Menachinol und der Weiterleitung der Elektronen an NapA beteiligt. Nitrat wird in *S. multivorans* zu Nitrit abgebaut, das anschließend durch eine Nitrit-Reduktase weiter zu Ammonium umgesetzt wird (Goris & Diekert, 2016).
In *S. multivoran* konnte die Beteiligung von Menachinon an der Fumarat-Respiration bereits experimentell gezeigt werden (Krauter, 2006), während sie für die Nitrat-Respiration nur auf Grund der vermuteten Ähnlichkeiten mit der Nitrat-Atmungskette von *W. succinogenes* angenommen wird. Welche Enzyme in *S. multivorans* bei der Fumarat- und Nitrat-Atmung an der Ausbildung der Protonenmotorischen Kraft beteiligt sind, wurde bislang nicht untersucht, jedoch kann auf Grund der hohen strukturellen Ähnlichkeiten der Formiat-Dehydrogenasen aus *S. multivorans* und *W. succinogenes* von einer elektrogenen Reduktion von Menachinon durch Formiat ausgegangen werden.

PCE-Respiration in *S. multivorans*

In *S. multivorans* erfolgt sowohl eine PCE-abhängige Regulation der Organohalid-Respiration als auch der Lokalisation der reduktiven Dehalogenase PceA. Wird *S. multivorans* mit anderen Elektronenakzeptoren als chlorierten Ethenen kultiviert, wird die Organohalid-Respiration langsam herunterreguliert bis sie nach circa 105 Generationen vollständig zum Erliegen kommt. Diese Langzeitregulierung beruht weder auf einem Verlust des *pceA*-Gens noch auf einer genetischen Veränderung in der Umgebung des *pceAB*-Operons. Die Organohalid-Respiration kann durch Kultivierung in Anwesenheit von PCE oder TCE innerhalb weniger Tage (etwa 3 Generationen) wieder induziert werden (John *et al.*, 2009; Goris & Diekert, 2016). Die gleiche Form der Langzeitregulierung konnte auch für *S. halorespirans* beobachtet werden (Goris & Diekert, 2016). Durch Lokalisationsstudien konnte zudem ein PCE-abhängiger Tat-Transport bzw. Lokalisation von PceA gezeigt werden. Wird *S. multivorans* für einige Generationen in Abwesenheit von PCE kultiviert, liegt PceA zum größten Teil in der nicht prozessierten Vorläuferform im Zytoplasma vor, während die reduktive Dehalogenase in Anwesenheit von PCE überwiegend in der physiologisch aktiven Form im Periplasma lokalisiert ist (John *et al.*, 2006; Goris & Diekert, 2016).

Obwohl für *S. multivorans* der Kenntnisstand über den genauen Aufbau der Organohalid-Atmungskette, insbesondere über die Komponenten, die für die Übertragung der Elektronen

zwischen Elektronendonor und der reduktiven Dehalogenase PceA verantwortlich sind, bisher unvollständig ist, konnte ein Modell für die Organohalid-Respiration in diesem Organismus erstellt werden. Das Modell der Organohalid-Atmungskette von *S. multivorans*, welches auf den aktuellsten Kenntnisstand beruht, ist in Abbildung 1-4 dargestellt und wird im Folgenden erläutert.

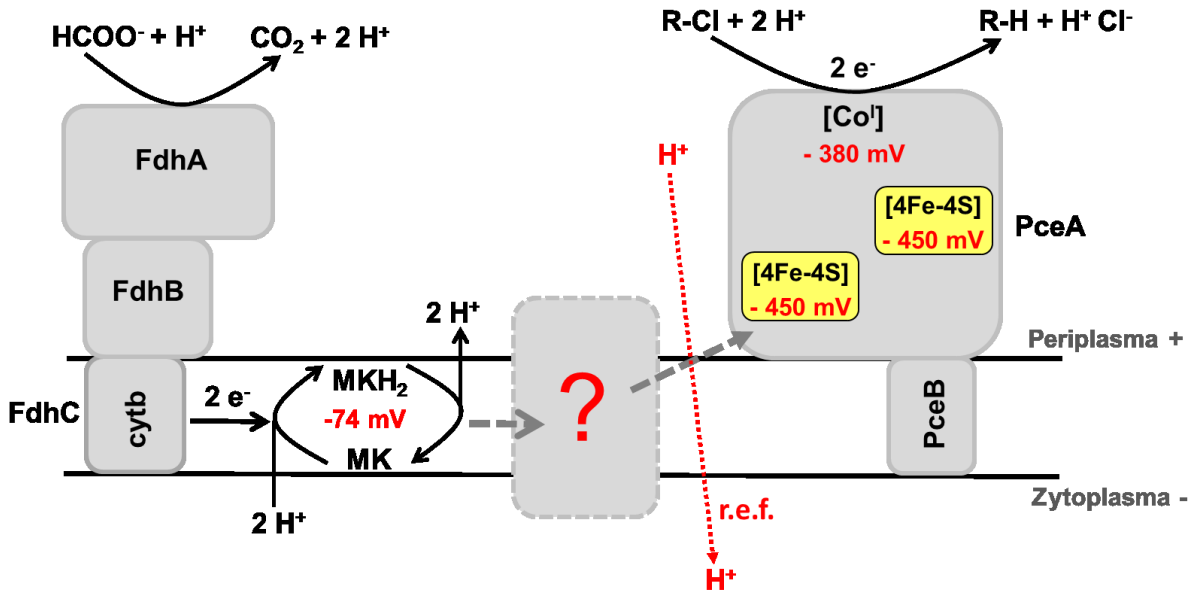

Abbildung 1-4: Modell der Organohalid-Atmungskette von *S. multivorans* mit Formiat als Elektronendonor. Fdh: Formiat-Dehydrogenase (FdhA, B, C), integriert in die Zytoplasmamembran durch die di-Häm Cytochrom *b* Untereinheit (FdhC); PceA: reduktive Dehalogenase, katalytisch aktive Untereinheit; PceB: putativer Membrananker; r.e.f.: revertierter Elektronentransport; MK/MKH_2: Menachinon/Menachinol; [Co^I]: [Co^{II}]/[Co^I]-Redoxpaar.

Das Schlüsselenzym der PCE-Respiration in *S. multivorans* ist die periplasmatisch lokalisierte reduktive Dehalogenase PceA (Abbildung 1-4), die PCE und TCE unter Energiegewinnung zu cis-1,2-DCE dechloriert (Abbildung 1-1). PceA enthält zwei [4Fe-4S] Cluster, für die ein Standard-Redoxpotential von ca. E_{SHE} = -450 mV wahrscheinlich ist (Siritanaratkul *et al.*, 2016). Bei dem Corrinoid-Kofaktor handelt es sich um Norpseudovitamin B_{12} (Kräutler *et al.*, 2003). Der [Co^{II}]/[Co^I]-Zustand des Corrinoid-Kofaktors wurde als reaktive Spezies identifiziert, die das Organohalid angreift (Miller *et al.*, 1996; Schubert & Diekert, 2016). Für das Redoxpaar [Co^{II}]/[Co^I] des enzymgebundenen Norpseudo-B_{12} wurde mittels EPR ein Mittelpunkt-Redoxpotential von E_{SHE} = -380 mV (pH 7,5) ermittelt (Kräutler *et al.*, 2003). Dieses Redoxpotential des Kofaktors war etwa 100 mV positiver als das Mittelpunkt-Potential des [CoII]/[CoI]-Paares des extrahierten und

gereinigten Norpseudovitamin B_{12} (E_{SHE} = -480 mV, pH 7,0) (Siebert, 2002), was auf einen Einfluss der Enzymumgebung auf das Redoxpotential schließen lässt (Schubert & Diekert, 2016). Für das isolierte Norpseudovitamin B_{12} wurde auch das Redoxpotential des [Co^{III}]/[Co^{II}]-Paares (E_{SHE} = - 140 mV; pH 7,0) mittels Redoxtitration bestimmt, wobei bislang für keine der getesteten Dehalogenasen die Bildung der [Co^{III}]- Oxidationsstufe beobachtet werden konnte. Ob diese eine Rolle beim Reaktionsmechanismus der reduktiven Dehalogenase spielt, bleibt daher unklar (Schubert & Diekert, 2016). PceA ist über den putativen Membrananker mit der Zytoplasmamembran assoziiert (Abbildung 1-4).

Als Elektronendonoren für die Organohalid-Respiration kann *S. multivorans* Formiat nutzen (Neumann *et al.*, 1994; Scholz-Muramatsu *et al.*, 1995). Das Formiat wird wie bereits beschrieben (siehe oben) durch die Formiat-Dehydrogenase oxidiert und die Elektronen wahrscheinlich auf Menachinon übertragen (Scholz-Muramatsu *et al.*, 1995; Schmitz & Diekert, 2004) (Abbildung 1-4). Die Beteiligung von Menachinon an der Elektronenübertragung innerhalb der Organohalid-Atmungskette wurde durch Inhibitionsversuche mit dem artifiziellen Semichinon-Analogon 2-n-Heptyl-4-hydroxychinolin-N-oxid (HQNO) getestet. Bei Verwendung hoher Konzentrationen an HQNO (80, 240 oder 320 nmol/mg Protein) konnten eine deutliche Hemmung der Organohalid-Atmung erzielt werden (Krauter, 2006), was die Hypothese von einer Menachinon-abhängigen PCE-Atmungskette stützt. Wie der Elektronentransport von Menachinon auf PceA erfolgt, ist bisher ungeklärt. Es gibt keine Hinweise auf eine direkte Interaktion zwischen dem membran-assoziierten PceA und dem Menachinon-Pool in der Zytoplasmamembran. Auch die Beteiligung von PceB an der Elektronenübertragung scheint unwahrscheinlich, da das Protein keine klassischen Bindemotive für Kofaktoren oder der Bindung von Metallionen aufweist und somit vom Fehlen elektronenübertragender Metall-Zentren, wie Eisen-Schwefel-Cluster oder Häm, ausgegangen werden muss (Schubert & Diekert, 2016). Die Beteiligung einer weiteren Komponente, die für die Oxidation des Menachinols und der Elektronenübertragung auf die terminale Reduktase PceA verantwortlich ist, ist wahrscheinlich (Abbildung 1-4).

Dient Menachinon, das ein Standard-Redoxpotential von E_{SHE}= -74 mv (bei pH 7,0) aufweist, als Elektronenüberträger innerhalb der PCE-Atmungskette, ergibt sich ein thermodynamisches Problem. Sowohl die zwei [4Fe-4S] Cluster von PceA, die höchst wahrscheinlich für die Übertragung der Elektronen auf den Corrinoid-Kofaktor verantwortlich sind als auch das [Co^{II}]/[Co^{I}]-Redoxpaar weisen deutlich negativere Redoxpoteniale auf (Abbildung 1-4). Die thermodynamisch ungünstige Reduktion von [Co^{II}] zu [Co^{I}] wird in *S. multivorans* sehr wahrscheinlich durch einen revertierten Elektronentransport (r.e.f.) angetrieben (Abbildung 1-4). Diese Annahme wird durch die Tatsache gestützt, dass die Organohalid-Respiration in *S. multivorans* durch Protonophore gehemmt werden kann (Miller *et al.*, 1996). Auch sprechen die

für *S. multivorans* bestimmten Wachstumsausbeuten (YS) bei Wachstum auf Wasserstoff und PCE (Scholz-Muramastu *et al.*, 1995) sowie die daraus berechnete ATP-Ausbeute (0,4 mol gebildetes ATP pro freigesetztem Cl^-; Miller *et al.*, 1996) für die Beteiligung eines revertierten Elektronentransports an der PCE-Atmung von *S. multivorans*.

Für die Freisetzung eines Halogenidions aus dem Substrat werden zwei Elektronen benötigt, folglich werden pro umgesetztem Organohalid zwei Protonen in das Exoplasma freigesetzt. Unter Berücksichtigung, dass 3 - 4 Protonen für die Synthese eines einzelnen ATP aus ADP plus P_i erforderlich sind (Weber & Senior, 2003; Mayer & Müller, 2014; Silverstein, 2014), wird weniger als ein ATP pro freigesetztem Halogenidion gebildet. Aus der Dehalogenierung von PCE über TCE zu cDCE, die durch Wasserstoffoxidation angetrieben wird, wird eine Gibbs freie Energie ($\Delta G^{\circ\prime}$) von 189 kJ pro Mol H_2 gewonnen (Holliger *et al.*, 1998). Diese Energie würde die Bildung von etwa 2,5 ATP ermöglichen, wenn 70 - 80 kJ/mol als notwendig erachtet werden, um ein ATP aus ADP und P_i in vivo zu bilden (Thauer *et al.*, 1977; Schink & Friedrich, 1994). Die niedrigen Wachstumsausbeuten von verschiedenen PCE-dechlorierenden OHRB mit Wasserstoff als Elektronendonator deuten jedoch auf einen niedrigeren Wert als ein ATP pro freigesetztem Halogenidion hin (Scholz-Muramastu *et al.*, 1995; Maymó-Gatell *et al.*, 1997). Mit dieser Beobachtung stimmt auch das für die PCE-Respiration von *Dehalobacter restrictus* bestimmte H^+/e^--Verhältnis von etwa 1 überein (Schumacher & Holliger, 1996). Ein ähnliches H^+/e^--Verhältnis wurde für die reduktive Dehalogenierung von 3-Chlorobenzoat durch *Desulfomonile tiedjei* gemessen (Mohn & Tiedjei, 1991).

1.5 Ziel der Arbeit

Bis heute ist nur wenig darüber bekannt, wie Energiekonservierung und reduktive Dehalogenierung in Organohalid-Respiration-betreibenden Mikroorganismen miteinander gekoppelt sind. Während die Schlüsselenzyme der Organohalid-Respiration, die reduktiven Dehalogenasen, sowie die Enzyme, die für die Oxidation der Elektronendonoren verantwortlich sind, schon lange bekannt sind, konnten bislang keine weiteren Proteine als Komponenten der Organohalid-Atmungsketten identifiziert werden. In der vorliegenden Arbeit sollten daher untersucht werden, ob sich weitere mögliche Komponenten der PCE-Atmungskette von *S. multivorans* finden und identifiziert lassen. Um dies zu realisieren, wurden sowohl das Genom als auch das Proteom des Organismus analysiert. Die Genomregion, in der das für die reduktive Dehalogenase kodierende Gen *pceA* lokalisiert ist, sollte charakterisiert werden, da Gene, die die gleiche funktionelle Aufgabe erfüllen, oft in unmittelbarer Nähe zueinander lokalisiert sind. Durch

einen Vergleich des sequenzierten Genoms von *S. multivorans* mit den Genomen nicht-dehalogenierender *Sulfurospirillum* Spezies sollten anschließend mögliche Komponenten der PCE-Atmungskette identifiziert werden. Für *S. multivorans* wurde eine PCE-abhängige Langzeitregulation der reduktiven Dehalogenase beschrieben (John *et al.*, 2009). Wird *S. multivorans* über längere Zeit in Abwesenheit von chlorierten Ethenen kultiviert, wird die Expression von *pceA* langsam herunterreguliert. Basierend auf diesem Ergebnis ist die Erzeugung von *S. multivorans* Zellen ohne PceA möglich. Durch einen Vergleich der Proteome von PceA-positiven und PceA-negativen Zellen sollten Proteine identifiziert werden, die der gleichen Regulation wie die reduktive Dehalogenase unterliegen und damit als mögliche Komponenten der PCE-Atmungskette identifiziert bzw. Ergebnisse aus der Genom-Analyse gestützt werden. Abschließend sollte die periplasmatische Komponente (Protein SMUL_1541) einer putativen Chinol-Dehydrogenase, die im Laufe der vorliegenden Arbeit als mögliche Komponente der PCE-Atmungskette identifiziert wurde, heterolog in *Escherichia coli* exprimiert und anschließend gereinigt werden.

Für *S. multivorans* wurde ein revertierter Elektronentransport als Voraussetzung für die Organohalid-Respiration beschrieben (Miller *et al*., 1996) und die Beteiligung von Menachinon an der Elektronenübertragung innerhalb der PCE-Atmungskette auf Grund von früheren Versuchen mit dem Semichinonanalogon 2-n-Heptyl-4-hydroxychinolin-N-oxid (HQNO; Scholz-Muramatsu *et al.*, 1995; Krauter, 2006) postuliert. Durch die Wiederholung von Versuchen vorangegangener Studien sollte in dieser Arbeit die Beteiligung eines revertierten Elektronentransports sowie von Menachinon an der Organohalid-Respiration von *S. multivorans* eindeutig bestätigt werden.

Die mikrobielle Dehalogenierung von PCE wurde unter anaeroben Bedingungen bei Redoxpotentialen kleiner -180 mV gezeigt (Kästner, 1991). Boden und Grundwasser, die oft mit chlorierten Ethenen verunreinigt sind, sind inhomogen in Bezug auf ihren Sauerstoffgehalt und schließen mikrooxische Zonen ein. Bisher wurde nicht geprüft, ob Bakterien in diesen Zonen potentiell zur reduktiven Dechlorierung fähig sind. In dieser Arbeit sollte daher weiterführend die Fähigkeit von *S. multivorans*, PCE unter oxischen Bedingungen reduktiv zu dechlorieren sowie die Proteomreaktion des Organismus auf Sauerstoff untersucht werden.

2. Übersicht zu den Manuskripten

Manuskript I

Insights into organohalide respiration and the versatile catabolism of *Sulfurospirillum multivorans* gained from comparative genomics and physiological studies

Tobias Goris, Torsten Schubert, Jennifer Gadkari, Tesfaye Wubet, Mika Tarkka, Francois Buscot, Lorenz Adrian and Gabriele Diekert

Veröffentlicht in: Environ Microbiol (2014), 16: 3562–3580

Die Aufklärung der Genomsequenz von *Sulfurospirillum multivorans* führte zu Einblicke in den vielseitigen Energiestoffwechsel dieses Organismus einschließlich der Organohalid-Atmung, die sich deutlich von der anderer Organohalid-atmenden Bakterien unterscheidet. Im Genom wurde eine 50 kbp große Region identifiziert, in der hauptsächlich Gene lokalisiert sind, die für Proteine kodieren, die entweder direkt (zum Beispiel die reduktive Dehalogenase PceA) oder indirekt (zum Beispiel für die *de novo* Biosynthese des Corrinoid-Kofaktors) an der Organohalid-Atmung beteiligt sind. In dieser Region wurden auch Gene gefunden, die eine putative Chinol-Dehydrogenase kodieren, die möglicherweise eine Rolle bei der Übertragung von Elektronen vom Chinon-Pool auf die reduktive PCE-Dehalogenase, das Schlüsselenzym der PCE-Atmung von *S. multivorans*, übertragen.

J. Gadkari führte die Wachstumsversuche mit unterschiedlichen Substraten sowie die Transkriptanalysen mittels RT-PCR und deren Auswertung durch.

(Eigenanteil: 10%)

Manuskript II

Proteomics of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates

Tobias Goris*, Christian L. Schiffmann*, Jennifer Gadkari*, Torsten Schubert, Jana Seifert, Nico Jehmlich, Martin von Bergen & Gabriele Diekert

Veröffentlicht in: Sci. Rep.(2015) 5, 13794; doi: 10.1038/srep13794

* geteilte Erstautorenschaft

In dieser Studie wurde eine umfassende vergleichende Proteomanalyse von *S. multivorans*, kultiviert mit verschiedenen Elektronendonoren und Elektronenakzeptoren, durchgeführt. Die Studie gab Aufschluss über die Komponenten, die direkt an der Organohalid-Atmung beteiligt sind sowie über Proteine, die bei der Reifung dieser Komponenten eine Rolle spielen. Des Weiteren zeigte die Studie die globale Reaktion auf PCE und andere Energiesubstrate im Epsilonproteobakterium *Sulfurspirillum multivorans*. Eine NapGH-ähnliche Chinol-Dehydrogenase wurde als die wahrscheinlichste Verbindung zwischen dem Chinon-Pool und der reduktiven Dehalogenase PceA identifiziert. In dieser Hinsicht ähnelt die PCE-Atmung von *S. multivorans* der Nap-vermittelten Nitratatmung wie sie für *Wolinella succinogenes* beschrieben ist. Die Proteine, beteiligt an der Norpseudovitamin B_{12}-Biosynthese, wurden ausschließlich in PCE-gewachsenen Zellen gefunden, was die entscheidende Rolle des Corrinoid-Kofaktor für die PCE-Atmung bestätigte. Diese Studie lieferte auch erste Einblicke in den Elektronentransfer von Pyruvat zu PCE einschließlich der möglichen Beteiligung einer chinonreduzierenden Pyruvat-Dehydrogenase und einer Pyruvat: Ferredoxin / Flavodoxin-Oxidoreduktase.

G. Diekert, T. Schubert, J. Seifert und M. von Bergen initiierten die Studie. T. Goris, G. Diekert und N. Jehmlich betreuten die Studie. T. Goris, C.L. Schiffmann und J. Gadkari verfassten das Manuskript und analysierten die Daten. C.L. Schiffmann führte die massenspektroskopischen Analysen sowie die Vorbereitung der Proteinproben durch; J. Gadkari führte die Nasslaborarbeiten mit den Zellen sowie die enzymatischen Analysen durch. T. Goris, C.L. Schiffmann, J. Gadkari, N. Jehmlich und G. Diekert diskutierten und interpretierten die Daten.

(Eigenanteil: 25%)

Manuskript III

Proteomic data set of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates

Tobias Goris*, Christian L. Schiffmann*, Jennifer Gadkari*, Lorenz Adrian, Martin von Bergen, Gabriele Diekert, Nico Jehmlich

Veröffentlicht in: *Data Brief* (2016) 8: 637-642.

* geteilte Erstautorenschaft

In diesem Manuskript werden keine neuen Daten vorgestellt. Das Manuskript diente der genaueren Beschreibung der Methoden verwendet in „Proteomics of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates“ (veröffentlicht in: Sci. Rep.(2015) 5, 13794; doi: 10.1038/srep13794). Des Weiteren sollte gezeigt werden dass die verwendeten Rohdaten spezifisch sind und diese bereitgestellt werden.

Das Manuskript enthält Zusatzinformationen zum Manuskript „Proteomics of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates” (veröffentlicht in: Sci. Rep. 5, 13794; doi: 10.1038/srep13794). Die Eigenanteile der einzelnen Autoren, entsprechen den bereits aufgeführten Anteilen für das Originalpaper. T. Goris, C.L. Schiffmann, J. Gadkari und N. Jehmlich verfassten das Manuskript.

(Eigenanteil: 25%)

Manuskript IV

Investigations on the tetrachloroethene respiratory chain of *Sulfurospirillum multivorans*

Jennifer Gadkari, Torsten Schubert, Tobias Goris, Sven Baumann, Gabriele Diekert

In Vorbereitung zur Veröffentlichung in: FEMS Microbiology Letters

In dieser Studie wurde gezeigt, dass *S. multivorans* zwei verschiedene Typen von Menachinon – Menachinon-6 und Methylmenachinon-6 – enthält, die als Elektronenüberträger in den verschiedenen Atmungsketten von *S. multivorans* fungieren. Dies wurde für die PCE-, Fumarat- und Nitrat-Atmung durch Hemmversuche mit dem Menachinon-Antagonisten HQNO (2n-Heptyl-4-hydroxychinolin-N-oxid) gezeigt. Des Weiteren wurde bewiesen, dass ein revertierter Elektronentransport benötigt wird um den Elektronentransport innerhalb der PCE-Atmungskette von *S. multivorans* anzutreiben, während die Fumarat- und Nitrat-Atmung davon unabhängig sind.

J. Gadkari führte die Experimente durch, analysierte die Daten und verfasste das Manuskript. T. Schubert, T. Goris und G. Diekert halfen beim Verfassen des Manuskripts. S. Baumann führte die massenspektroskopischen Analysen durch. J. Gadkari, T. Schubert, T. Goris und G. Diekert konzipierten und gestalteten die Studie.

(Eigenanteil: 80%)

Manuskript V

Purification of the periplasmatic component of a putative quinol dehydrogenase involved in tetrachloroethene respiration in *Sulfurospirillum multivorans*

Jennifer Gadkari, Torsten Schubert, and Gabriele Diekert

In Vorbereitung zur Veröffentlichung in: Protein Expression and Purification

Eine putative Chinol-Dehydrogenase, bestehend aus einer membranintegralen Komponente (SMUL_1542) sowie einer periplasmatisch lokalisierten Komponente (SMUL_1541), ist wahrscheinliche an der Elektronenübertragung innerhalb der PCE-Atmungskette von *S. multivorans* beteiligte. In dieser Studie wurde die periplasmatische, Eisen-Schwefel-Cluster-enthaltende Komponente SMUL_1541 (als C-terminale Fusion mit Strep-Tag II) heterolog in *Escherichia coli* exprimiert und anschließend gereinigt. Nach Rekonstitution der Eisen-Schwefel-Cluster enthielt das gereinigte Protein 8 mol Eisen pro Mol Enzym, was 50% des vorhergesagten Eisengehalts ausmachte.

J. Gadkari führte die Experimente durch, analysierte die Daten und verfasste das Manuskript. T. Schubert und G. Diekert halfen beim Verfassen des Manuskripts. J. Gadkari, T. Schubert und G. Diekert konzipierten und gestalteten die Studie.

(Eigenanteil: 90%)

Manuskript VI

Reductive tetrachloroethene dehalogenation in the presence of oxygen by *Sulfurospirillum multivorans*: Physiological studies and proteome analysis

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Eingereicht zur Veröffentlichung bei: FEMS Microbiology Ecology am 23.06.2017

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In dieser Studie wurde die Fähigkeit des mikroaeroben Organismus *Sulfurospirillum multivorans* zur reduktiven Dehalogenierung von Tetrachlorethen (PCE) in Anwesenheit von Sauerstoff untersucht. Da es bislang nur wenige Informationen zur physiologischen Reaktion von mikroaeroben Organohalid-atmenden Bakterien oder Epsilonproteobakterien auf Sauerstoff gibt, wurde des Weiteren die zelluläre Antwort des Organismus auf Sauerstoff durch eine Proteomanalyse untersucht. Es konnte gezeigt werden, dass *S. multivorans* PCE in Gegenwart von Sauerstoffkonzentrationen unter 0,5% reduktiv dechlorieren kann. Als Stressantwort auf Sauerstoff wurden in *S. multivorans* hauptsächlich zwei Enzyme, eine Superoxid-Reduktase und eine Hydroperoxid-Reduktase hochreguliert. Die Ergebnisse dieser Studie sind wichtig für Untersuchungen zur reduktiven Dehalogenierung in oxisch-anoxischen Zonen von PCE-kontaminierten Standorten.

J. Gadkari führte die Experimente zur reduktiven Dechlorierung von Tetrachlorethen in Anwesenheit von Sauerstoff sowie die enzymatischen Analysen und die Auswertung der gaschromatographischen Analysen durch. J. Gadkari half C.L. Schiffmann bei der Kultivierung der Zellen für die Proteomanalysen. C.L. Schiffmann führte die massenspektroskopischen Analysen sowie die Vorbereitung der Proteinproben durch. R. Rubick führte die Experimente zur reduktiven Dechlorierung von Tetrachlorethen in Abhängigkeit vom Redoxpotential durch. J. Gadkari, T. Goris und C.L. Schiffmann verfassten das Manuskript und analysierten die Daten. J. Gadkari, T. Goris, C.L. Schiffmann und G. Diekert diskutierten und interpretierten die Daten.

(Eigenanteil: 30%)

3. Manuskripte

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3.6 Manuskript VI: Reductive tetrachloroethene dehalogenation in the presence of oxygen by *Sulfurospirillum multivorans*: Physiological studies and proteome analysis

3.1 Manuskript I

environmental microbiology

Environmental Microbiology (2014) **16**(11), 3562–3580 doi:10.1111/1462-2920.12589

Insights into organohalide respiration and the versatile catabolism of *Sulfurospirillum multivorans* gained from comparative genomics and physiological studies

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Summary

***Sulfurospirillum multivorans*, a free-living ε-proteobacterium, is among the best studied organisms capable of organohalide respiration. It is able to use several halogenated ethenes as terminal electron acceptor. In this report, the complete genome sequence of *S. multivorans* including a comparison with genome sequences of two related non-dehalogenating species, *Sulfurospirillum deleyianum* and *Sulfurospirillum barnesii*, is described. The 3.2 Mbp genome of *S. multivorans* revealed a ~ 50 kbp gene region encoding proteins required for organohalide respiration and corrinoid cofactor biosynthesis. This region includes genes for components not detected before in organohalide-respiring organisms. A transcript analysis of genes coding for some of these proteins indicates the involvement of a putative quinol dehydrogenase in organohalide respiration. The presence of genes encoding a variety of oxidoreductases reflects the organism's metabolic versatility. This was confirmed by growth studies with different electron acceptors including perchlorate and several sulfur-containing compounds. A comparison with other ε-proteobacteria indicates horizontal acquisition of many genes in the *S. multivorans* genome, which might be the basis of the bacterium's catabolic flexibility.**

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Introduction

Halogenated organic compounds (organohalides) are environmentally harmful and/or toxic, often alongside being carcinogenic (Henschler, 1994). Some of them are among the most persistent anthropogenic compounds found in the environment (Häggblom and Bossert, 2003). Whereas many organohalides are produced naturally through biotic or abiotic processes (Gribble, 2003; Wagner *et al.*, 2009), a lot of these compounds are abundant in contaminated environments as remnants of agricultural (pesticides) or industrial processes (Warren *et al.*, 2003; Breivik *et al.*, 2004). Organohalides can be dehalogenated abiotically (Tobiszewski and Namiesnik, 2012) or enzymatically under oxic (Fetzner, 1998) or anoxic conditions (Smidt and de Vos, 2004), under which reductive dehalogenation is a frequently used biological process.

The most widely known form of microbial reductive dehalogenation is the use of organohalides as terminal electron acceptors in anaerobic respiration, a process referred to as organohalide respiration. Many different compounds may serve as substrates in reductive dehalogenation, including highly toxic environmental pollutants such as dioxins and polychlorinated biphenyls (Quensen *et al.*, 1988; Bunge *et al.*, 2003). Some of the most abundant halogenated compounds are chlorinated ethenes (Fetzner, 1998), which are highly persistent in the environment. The dehalogenation of tetrachloroethene (PCE) to trichloroethene (TCE) and of TCE to *cis*-1,2-dichloroethene (cDCE) is carried out by several organisms including members of the δ- and ε-*Proteobacteria* and of the *Firmicutes* (Maphosa *et al.*, 2010; Hug *et al.*, 2013). Only a few *Dehalococcoides mccartyi* strains have been shown to completely dechlorinate chlorinated ethenes to the non-toxic ethene (Maymó-Gatell *et al.*, 1997; Cichocka *et al.*, 2010; Pöritz *et al.*, 2013).

The only ε-proteobacteria described so far to perform organohalide respiration are found in the genus *Sulfurospirillum* (Scholz-Muramatsu *et al.*, 1995; Luijten

et al., 2003). These organisms utilize chlorinated ethenes as terminal electron acceptors (Neumann *et al.*, 1994). Dechlorinating and non-dechlorinating members of the genus *Sulfurospirillum*, grouped into the family *Campylobacteraceae*, are found in various habitats including sediments and soils as well as marine environments, oil reservoirs and sewage plants (Schumacher *et al.*, 1992; Finster *et al.*, 1997; Stolz *et al.*, 1999; Campbell *et al.*, 2001; Jensen and Finster, 2005; Hubert and Voordouw, 2007; Kodama *et al.*, 2007). The species under investigation in our study, *Sulfurospirillum multivorans*, has been isolated from activated sludge of a wastewater treatment plant (Scholz-Muramatsu *et al.*, 1995). *Sulfurospirillum multivorans* uses a wide range of electron donors (e.g. H_2, formate, pyruvate and lactate) and electron acceptors (e.g. PCE, TCE, fumarate, nitrate, selenate and arsenate) for anaerobic respiration (Scholz-Muramatsu *et al.*, 1995; Luijten *et al.*, 2004). Additionally, it disproportionates fumarate and grows very slowly with pyruvate as sole substrate. It is among the best studied organisms carrying out organohalide respiration (Miller *et al.*, 1996; Holliger *et al.*, 1998). The key enzyme of organohalide respiration in this organism, the PCE reductive dehalogenase (PceA), was isolated and characterized and the corresponding gene was sequenced (Neumann *et al.*, 1996; 1998). PceA, like nearly all described reductive dehalogenases (Hug *et al.*, 2013), is a corrinoid and iron-sulfur cluster containing enzyme. It dechlorinates PCE via TCE to *c*DCE and tribromoethene to vinyl bromide, at least the latter reaction being mediated probably via a radical mechanism (Schmitz *et al.*, 2007; Ye *et al.*, 2010). PceA is localized in the periplasm (John *et al.*, 2006), anchored most probably to the cytoplasmic membrane via a small hydrophobic protein, PceB (Neumann *et al.*, 1998). The PceA enzyme harbours a unique type of corrinoid cofactor not found in other organohalide-respiring bacterial classes to date, a norpseudo-B_{12} (Kräutler *et al.*, 2003), which is essential for reductive dechlorination (Siebert *et al.*, 2002). This cofactor is synthesized *de novo* by *S. multivorans* (Keller *et al.*, 2013). The expression of the *pceA* gene undergoes a long-term regulation (John *et al.*, 2009), the mechanism of which is unknown.

Components of the organohalide respiratory (OHR) chain apart from the reductive dehalogenase are still not identified and the variability of the catabolism of *S. multivorans* is widely unexplored. The genomic environment of the *pce* genes was not known, so that information about genes in close vicinity, which are often functionally linked, was missing. Therefore, we sequenced the genome of *S. multivorans* to compare it with the genomes of its closest non-dehalogenating relatives, which allowed for the identification of components possibly involved in organohalide respiration. Additionally, the genome sequence revealed the genes responsible for *de novo* norpseudo-B_{12} biosynthesis and genetic elements presumably playing a role in the regulation of PCE dechlorination in *S. multivorans*. Because the key components of the OHR chain are not encoded in the non-dechlorinating ε-*Proteobacteria*, the possibility and the underlying mechanisms of horizontal gene acquisition is discussed. Furthermore, we draw a picture of the highly variable catabolic lifestyle of the *Sulfurospirillum* genus in general and *S. multivorans* in particular including physiological studies with different electron acceptors.

Results and discussion

General features of the genome of S. multivorans *and comparison with other* Sulfurospirillum *genomes*

The genome of *S. multivorans* (GenBank accession no. CP007201) consists of one circular chromosome with 3 175 729 bp containing 3301 open reading frames (ORFs). The total GC content is 40.9%. Two complete rRNA clusters and 45 tRNAs were detected. The genome is the second largest complete ε-proteobacterial one described so far, the largest being the 3.192 Mbp genome of *Arcobacter nitrofigilis* (Pati *et al.*, 2010); the draft genome of *Arcobacter* sp. CAB (Carlstrom *et al.*, 2013) points to a genome of about 3.5 Mbp, the current maximum for ε-*Proteobacteria*. Compared with the complete genomes of its closest relatives, *Sulfurospirillum deleyianum* (Sikorski *et al.*, 2010b) and *Sulfurospirillum barnesii* (GenBank accession no. CP003333), the *S. multivorans* genome is larger by approximately 0.8 and 0.5 Mbp respectively. Two draft genomes of marine *Sulfurospirillum* species are available on the Genome Portal of the Department of Energy Joint Genome Institute. The genome of *Sulfurospirillum* strain AM-N (Portal of the Department of Energy Joint Genome Institute, IMG object ID 2502171155) is about 2.3 Mbp in size, while that of *Sulfurospirillum arcachonense* (GenBank accession no. JFBL00000000) is about 2.7 Mbp. The additional size of the *S. multivorans* genome to a large part (~ 150 kbp) is made up by a region spanning the coding sequences SMUL_1716 to SMUL_1898 (phage-containing region between 1.6–1.8 Mbp in Fig. 1). Of the 182 ORFs in this region, only eight produce bidirectional BLAST hits to any of the four other *Sulfurospirillum* spp. genomes. These eight ORFs are scattered around the whole 150 kbp region. This region has a 6% lower GC content than the genome average and a high percentage of ORFs, which either cannot be assigned to any function or are related to DNA binding and repair, plasmid stabilization or genetic mobilization. The genes related to genetic mobilization include several ORFs presumably encoding proteins similar to the Tra system (Anthony *et al.*, 1999). Some single phage proteins were found in that region and an analysis with the phage finder software PHISPY (Akhter

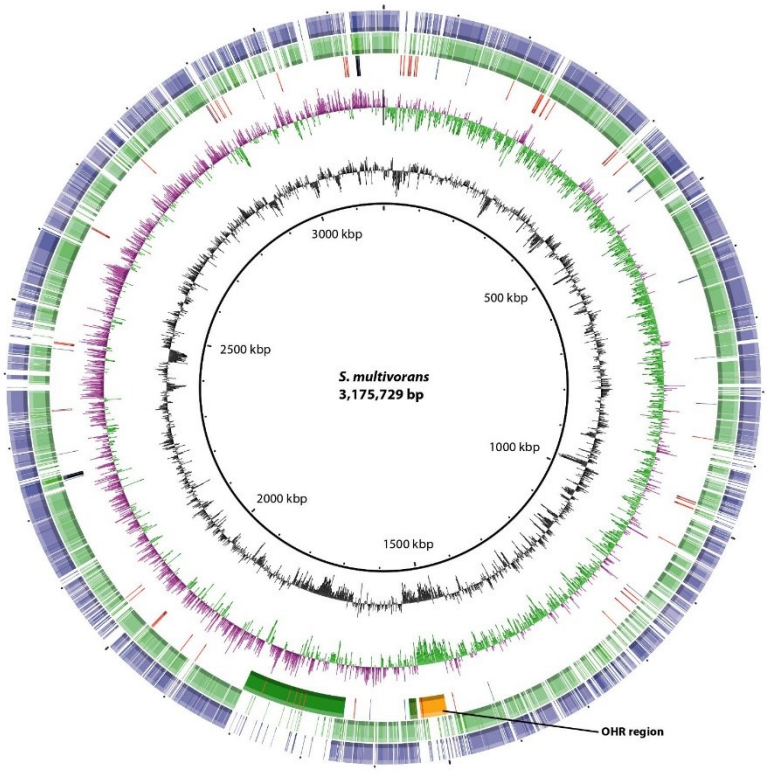

Fig. 1. Circular representation of the genome sequence of *S. multivorans* in comparison with the genomes of *S. deleyianum* (green) and *S. barnesii* (blue). Rings from inside to outside: (i) GC content (black), (ii) GC skew (green/pink including Ori marked in black), (iii) selected regions in the *S. multivorans* genome (tRNA genes: blue; rRNA genes: black; transposase and integrase genes: red; phage-like regions: green; OHR region: orange), (iv) BLAST comparison with *S. deleyianum* (light green) and (v) BLAST comparison with *S. barnesii* genome sequence (blue). Nucleotide sequence identities from 30% to 50% are in light colour, 50–70% in intermediate colour, more than 70% in dark colour. The image was generated with BRIG (Alikhan *et al.*, 2011).

et al., 2012) revealed the presence of a putative prophage spanning the whole 150 kb region. Not far away from that region, a second putative phage was found with PHAST (Zhou *et al.*, 2011), showing distant similarity to *Enterobacteria* phage P4 (NCBI reference sequence NC_001609). Essential phage genes are missing from the corresponding phage sequence found in the *S. multivorans* genome. Only the genes encoding transposases (SMUL_1588-1589), a phage integrase (SMUL_1595), a fibre protein (SMUL_1598) and four other hypothetical phage-like proteins were found to be located between two att-sites and one tRNA gene, known for being a preferred integration site for phages (Reiter *et al.*, 1989). Apart from these distinct parts of the genome, other gene regions of *S. multivorans*, which are not found in other *Sulfurospirillum* spp. genomes, are much smaller and scattered all over the genome. The large number of transposases in comparison with other ε-proteobacteria (Table 1) indicate genome rearrangement and horizontal gene acquisition. Of all protein coding sequences in the *S. multivorans* genome, 26.2% have no representatives in the 3 best BLAST hits to the other four *Sulfurospirillum* genome sequences (see Table 2).

One clustered regularly interspaced short palindromic repeats (CRISPR) region of about 10 kbp was found in the genome of *S. multivorans*, with 146 spacers separated by direct repeats of 30 bp. The large number of spacers is unusual for ε-*Proteobacteria*, many of them containing only a few (1–10) spacers (Rousseau *et al.*, 2009). This finding suggests that *S. multivorans* got in contact with a high number of different phages. *Sulfurospirillum deleyianum* and *S. barnesii* have 109 and

Table 1. Overall genome features of *S. multivorans* and other ε-*Proteobacteria*.

	Sulfurospirillum					
	multivorans	*deleyianum*	*barnesii*	*W. succinogenes*	*C. jejuni*	*H. pylori*
Size (kbp)	3176	2306	2510	2110	1641	1668
ORFs	3301	2346	2565	2098	1705	1624
GC content	41%	39%	39%	48%	31%	39%
tRNAs	45	43	41	40	44	36
rRNAs	2	3	2	3	3	2
Transposases	43	18	11	17	2	8

Genome sequences from: *W. succinogenes* (Baar *et al.*, 2003), *C. jejuni* (Parkhill *et al.*, 2000), *H. pylori* (Tomb *et al.*, 1997), *S. deleyianum* (Sikorski *et al.*, 2010b) and *S. barnesii* (GenBank accession no. CP003333).
ORFs, open reading frames including RNA genes; Transposases, number of putatively functional transposases.

41 spacers respectively. Eight *cas* genes (SMUL_1140-1148) are located upstream of the CRISPR region. The structure of this *cas* gene cluster resembles the genetic structure the *cas* gene cluster found in *S. barnesii* except a gene coding for a prophage antirepressor-like protein (SMUL_1145). According to the current Cas-CRISPR classification (Makarova *et al.*, 2011), *S. multivorans* harbours a type I system.

Among the isolated ε-proteobacterial species, *S. multivorans* and the closely related *Sulfurospirillum halorespirans* are the only representatives unambiguously shown to be capable of organohalide respiration (Neumann *et al.*, 1994; Luijten *et al.*, 2003). A 50 kbp region in the *S. multivorans* genome harbours most genes predicted to be involved in the OHR chain. The genes in this OHR region have no orthologues [bidirectional blast hits with more than 50% amino acid sequence identity with a ratio of the coding sequence length between 0.9 and 1.1, as defined by Kube and colleagues (2005)] in the genomes of the non-dechlorinating *S. deleyianum* and *S. barnesii*, as opposed to the surrounding genomic regions. The OHR region is located nearly opposite to the origin of replication (Fig. 1). Most of the region is covered by genes encoding proteins involved either in PCE reductive dehalogenation or *de novo* corrinoid biosynthesis. Such a close clustering of genes for reductive dehalogenation and *de novo* corrinoid biosynthesis has not been found in any other organohalide-respiring organism [e.g. *Desulfitobacterium hafniense* strains (Nonaka *et al.*, 2006; Kim *et al.*, 2012) or *Geobacter lovleyi* SZ (Wagner *et al.*, 2012)].

The reductive dehalogenase gene region

Downstream of the previously described *pceAB* genes (Neumann *et al.*, 1998), a second reductive dehalogenase gene cluster was found (Fig. 2). The second cluster also contains the genes coding for a reductive dehalogenase structural protein (RdhA, 70% amino acid sequence identity to PceA) and for a small hydrophobic protein (RdhB, 90% amino acid sequence identity to PceB). The N-terminal Tat (twin arginine translocation) signal peptide and two iron-sulfur cluster binding motifs are conserved in PceA and RdhA. For both B-protein sequences, two transmembrane helices were predicted. The transcript of the *rdhA* gene was not detected in significant amounts, neither in PCE-grown cells nor in fumarate-grown cells with repressed *pceA* transcription (Fig. S1). Other RdhAs with 100% amino acid sequence identity to RdhA of *S. multivorans* are found in *S. halorespirans* (Luijten *et al.*, 2003) as well as in a PCE-dechlorinating enrichment culture containing *Sulfurospirillum* spp. (Buttet *et al.*, 2013). The function of this highly conserved RdhA in PCE-respiring *Sulfurospirillum* spp. remains to be unravelled. Downstream of each *rdh* gene cluster, genes encoding a two-component regulatory system, comprising a putative histidine-protein kinase (HPK) and a putative response regulator (RR), were found (Fig. 2). The *in silico* topology analysis predicted seven transmembrane helices for each HPK. The two HPK sequences displayed 38% amino acid sequence identity while the RR sequences shared 55% identity. Both types of proteins have their closest relatives in the ε-proteobacterial *Sulfurimonas* genus (29% amino acid sequence identity for HPK, 40% for RR). The BLAST analysis of theputative periplasmic

Table 2. *S. multivorans* genome BLAST searches against the NCBI refseq protein database and the draft genomes of *S. arcachonense* and *Sulfurospirillum* strain AM-N.

Top 3 BLAST hits	Number of genes	% of genome
Orphans	159	4.9
Other bacterial classes	331	10.2
Other ε-Proteobacterial genera	357	11.0
Sulfurospirillum spp.	2454	73.9

Shown are the numbers of CDS that are orphans (genes with no BLAST hits); genes that have only other bacterial classes in the top three BLAST hits; genes with ε-proteobacterial, but no *Sulfurospirillum* hits in the top three BLAST hits; and genes with best hits against at least one of the three *Sulfurospirillum* genomes. The e-value for orphan analysis was set to 1e-04. Top three BLAST hits were chosen to include hits with very similar BLAST scores.

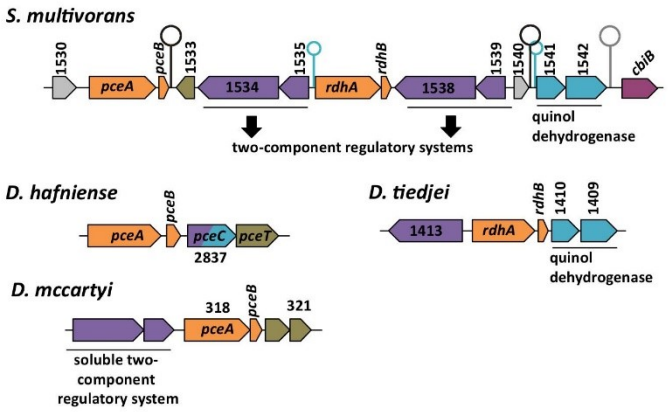

Fig. 2. PceA gene cluster from *S. multivorans* in comparison with *pce* gene clusters of other PCE-respiring organisms [*Desulfitobacterium hafniense* strain Y51 (Nonaka *et al.*, 2006), *Desulfomonile tiedjei*, *Dehalococcoides mccartyi* strain 195 (Seshadri *et al.*, 2005)]. Orange: reductive dehalogenase genes; violet: regulator proteins; olive: accessory proteins; turquoise: redox enzymes; grey: unknown function; grey stem and loop: weak transcriptional terminators (calculated $\Delta G > -10$ kJ mol^{-1}); black stem and loop: strong transcriptional terminators (calculated $\Delta G < -10$ kJ mol^{-1}); light blue stem and loop: translational attenuators.

N-terminal domain of the HPKs, probably responsible for substrate binding, delivered no hits, pointing to the presence of a novel signal recognition site presumably adapted to halogenated ethenes.

While the *rdhAB* gene cluster is directly followed by two genes encoding the two-component regulator, a small gene (SMUL_1533), encoding a protein with unknown function (Fig. 2), is inserted between the *pceAB* gene cluster and the genes encoding the two-component regulator. The gene product shows similarity to proteins involved in iron-sulfur cluster maturation (NifU and IscU); hence, it might have a function in correct insertion of the iron-sulfur clusters into PceA.

Downstream of the two *rdh* gene clusters and upstream of the corrinoid biosynthesis gene cluster, two ORFs were identified encoding a putative membrane-bound enzyme complex similar to NapGH/NosGH quinol dehydrogenases (Kern and Simon, 2008; Simon and Klotz, 2013). Deduced from the vicinity of its genes to the *rdh* gene cluster, a function in the PCE respiratory chain is feasible. The protein complex might serve as an electron carrier between the menaquinone pool and PceA, analogous to the function of the NapGH complex in periplasmic nitrate reduction of *Wolinella succinogenes* (Kern and Simon, 2008). Genes encoding enzymes involved in the biosynthesis of menaquinones via the futalosine route (Hiratsuka *et al.*, 2008) are encoded in *S. multivorans*, while the enzymes of the ubiquinone pathway are not.

The *napG*/*nosG* gene homologue (SMUL_1541) encodes a protein containing an N-terminal Tat signal peptide and is therefore considered to be localized in the periplasm. The amino acid sequence identity of SMUL_1541 to NapG of *W. succinogenes* is about 30%. All of the 16 cysteines putatively binding four [4Fe-4S] clusters in NapG are conserved in SMUL_1541. The NapH homologue (SMUL_1542, 25% sequence identity to NapH) was predicted to contain four membrane-spanning helices when subjected to topology prediction. Eight cysteines forming binding motifs for two [4Fe-4S] clusters are conserved in SMUL_1542 as well. The [4Fe-4S] clusters are putatively located in the cytoplasmic part of the protein as shown for the *Escherichia coli* NapH, where these cytoplasmically oriented [Fe-S] cubanes might monitor the cellular redox state (Brondijk *et al.*, 2004). Upstream of the quinol dehydrogenase genes, a gene is localized that encodes a small putative membrane protein (about 12 kDa) with three predicted transmembrane helices and unknown function. Another genome, in which *rdhAB* genes are directly adjacent to genes encoding a putative quinol dehydrogenase (Desti_1409-1410), is that of *Desulfomonile tiedjei* (GenBank accession no. CP003360), a 3-chlorobenzoate-respiring δ-proteobacterium (see Fig. 2). The corresponding RdhA (encoded by Desti_1412) exhibits the highest sequence identity to PceA (39%) and RdhA (42%) of *S. multivorans* outside the genus *Sulfurospirillum*. *Desulfomonile tiedjei* also dechlorinates PCE (Cole *et al.*, 1995; Townsend and Suflita, 1996), but at a slow rate, and the reductive PCE dechlorination is not coupled to growth of the organism. The putative quinol dehydrogenases of *S. multivorans* and *D. tiedjei* form a distinct clade in the phylogenetic tree of quinol dehydrogenases (Fig. S2).

Directly upstream of *pceA* in *S. multivorans*, a gene (SMUL_1530) encoding an alkylhydroperoxidase-like protein is located. An alkylhydroperoxidase was shown to be protective against oxidative stress in *Mycobacterium tuberculosis* (Koshkin *et al.*, 2003). The function of the SMUL_1530 gene product might be the protection of the premature form of PceA from oxidative stress in the cytoplasm. Upstream of this peroxidase gene, several gene clusters (e.g. *rib* gene cluster) were found, which cannot

be assigned to a function in organohalide respiration or the maturation of any of the proteins involved.

Four transcriptional terminators and two translational attenuators were found close to the *pceA* gene, while riboswitches were not detected in the OHR region. Directly downstream of the *rib* gene cluster and upstream of the peroxidase gene, a weak terminator structure was found (Fig. 2), indicating that the *rib* genes are transcribed independently of the reductive dehalogenase gene clusters. Downstream of the *pceAB* genes, a transcriptional terminator is located (Fig. 2; Neumann *et al.*, 1998). The *rdhAB* gene cluster mirrors this pattern, but unlike in the *pceAB* gene cluster, a translational attenuator is found upstream of *rdhA*. One further translational attenuator gene was found upstream of the quinol dehydrogenase genes. Adjacent to the respective genes, a transcriptional terminator sequence was found; such a terminator is also present upstream of the quinol dehydrogenase genes. All of these findings indicate a distinct expression control of the reductive dehalogenases, the quinol dehydrogenase and the corrinoid biosynthesis genes.

De novo *corrinoid biosynthesis*

Sulfurospirillum multivorans is able to synthesize corrinoids *de novo*; hence, an addition of corrinoids to the growth medium is not necessary when the organism is grown with PCE (Keller *et al.*, 2013). Corrinoid biosynthesis starts from uroporphyrinogen III, which is most probably produced in *S. multivorans* from glutamate via 5-aminolevulinic acid using the C_5-pathway (Beale *et al.*, 1975). The gene cluster coding for corrinoid biosynthesis from uroporphyrinogen III contains 26 ORFs (SMUL_1543-1568, Fig. 3) including genes encoding an ABC transporter (*btuCDF*). With one exception, the corrinoid biosynthesis gene cluster includes all genes necessary for the corrinoid biosynthesis under anoxic conditions (Warren *et al.*, 2002). A gene encoding an ATP:cob(I)alamin adenosyltransferase (*cobA*-, *pduO*- or *eutT*-like) is not present in the *S. multivorans* genome, implying either a lack of corrinoid adenosylation or the use of an analogous reaction mediated by a different protein.

Up-to-date ε-*Proteobacteria* were not known to synthesize corrinoids *de novo* (Zhang *et al.*, 2009). The comparison of the currently available ε-proteobacterial genomes of 48 different species [GOLD database (Pagani *et al.*, 2012) as of March 2014] revealed the presence of *de novo* corrinoid biosynthesis gene clusters exclusively in *S. multivorans*, *S. barnesii*, *S. arcachonense*, *Sulfurospirillum* strain AM-N and *A. nitrofigilis*. However, the gene arrangements in the latter four species differ from that of the corrinoid biosynthesis gene cluster in *S. multivorans*. The amino acid sequence identity of the proteins encoded by the gene clusters of these organisms is only 25–45% to those of *S. multivorans*. A higher sequence identity (between 40–80%) and a higher grade of synteny to the corrinoid biosynthesis gene cluster of *S. multivorans* is found in several *Fusobacteria* (*Firmicutes*), which do not contain reductive dehalogenase genes. This especially applies to gene clusters from the fusobacterial species *Sebaldella termitidis* and *Ilyobacter polytropus* (Fig. 3). Three unusual ORFs are located within the corrinoid biosynthesis gene cluster of *S. multivorans* and are also present in the genome of *I. polytropus* (Sikorski *et al.*, 2010a). SMUL_1548 and SMUL_1567 encode two small, cysteine-rich proteins. SMUL_1565 is an *msbA*-like gene and might encode for a lipid transport system. A role in corrinoid biosynthesis has not yet been assigned to the respective gene products. The corrinoid produced by *I. polytropus* when cultivated on hydroxybutyrate (Stieb and Schink, 1984) was extracted and identified via LC-MS as pseudo-B_{12} (Fig. S3). Pseudo-B_{12} contains a methyl group at position 176 in the linker moiety of its nucleotide loop, which is absent in norpseudo-B_{12} of *S. multivorans* (Kräutler *et al.*, 2003). From this result, we assume that the structural difference between these two corrinoids might be due to the function of a gene product outside the set of orthologous genes present in both organisms. No

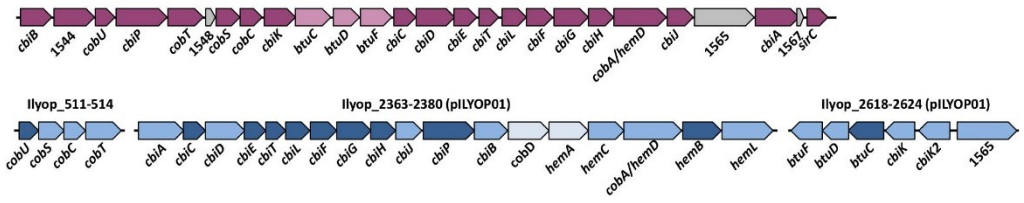

Fig. 3. Corrinoid biosynthesis gene cluster of *S. multivorans* (top) and the corrinoid biosynthesis gene clusters from *I. polytropus* (bottom). Numbers given are the locus tags of the respective genes (SMUL_XXXX for *S. multivorans*, Ilyop_XXXX for *I. polytropus*; pILYOP01 denotes the plasmid of *I. polytropus*). Purple ORFs: gene products are involved in *de novo* corrinoid biosynthesis; pink ORFs: encode transport proteins; grey ORFs: gene products with unknown functions. The orthologous genes in *I. polytropus* are coloured depending on their amino acid sequence identity with their respective orthologues found in the genome of *S. multivorans*. Dark blue ORFs: more than 50%; blue ORFs: 30–50%; light blue: less than 30%.

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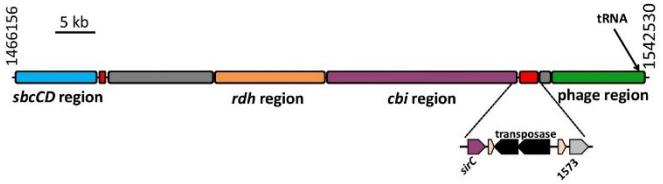

Fig. 4. Arrangement of the OHR region and surrounding elements putatively involved in horizontal gene transfer. The position in the genome is given in bp at the edges of the region. Zoom-in: the *tetR* gene downstream of the corrinoid biosynthesis cluster disrupted by a transposase. Transposase genes are shown in red. Regions putatively not involved in OHR or gene transfer are depicted in grey. *sbcCD* region: nuclease system; *rdh* region: reductive dehalogenase homologous gene region; *cbi* region: corrinoid biosynthesis cluster.

significant BLAST hits (> 30%) in fusobacterial genomes were obtained with the sequences of two ORFs located at the beginning of the corrinoid biosynthesis gene cluster (Fig. 3), namely *cbiB*, the gene for the adenosyl cobinamide phosphate synthase, and SMUL_1544, encoding a protein with distant relationship to the threonine phosphate decarboxylase CobD. Homologues of these gene products were shown to be involved in the generation and incorporation of the linker moiety connecting the corrin ring with the lower ligand base in the corrinoid (Warren *et al.*, 2002). CbiB shows highest similarities (amino acid sequence identity of 54%) to the corresponding gene products of *Sulfurimonas autotrophica*. (ε-proteobacterium), in which CbiB might play a role in corrinoid salvaging. The gene product of SMUL_1544 displays very low amino acid sequence identity (24%) to biochemically characterized threonine phosphate decarboxylases (CobD) (for a sequence alignment see Fig. S4). It is more closely related to aminotransferase sequences of *Campylobacter jejuni* (e.g. Cj1436c, amino acid sequence identity of 46%), the products of which might be involved in the modification of lipopolysaccharides in the *C. jejuni* capsule (Karlyshev *et al.*, 2000). The gene product of SMUL_1544 might be involved in the production of ethanolamine for incorporation into the linker moiety of norpseudo-B_{12}. A function as a putative serine phosphate decarboxylase is feasible.

Possible acquisition of the OHR region by horizontal gene transfer

The GC content as well as the GC skew of the OHR region are deviant from the residual *S. multivorans* genome (see Fig. 1), a fact that indicates horizontal acquisition of this genetic material. The GC content and GC skew of the reductive dehalogenase region and the corrinoid biosynthesis cluster are similar, but because the corrinoid biosynthesis genes of *S. multivorans* are closely related to those present in *Fusobacteria* (devoid of reductive dehalogenase genes), the acquisition of the OHR genes and of the corrinoid biosynthesis genes might have occurred independently. Further evidence for horizontal gene transfer of components of the OHR region is available. The two putative phage sequences in *S. multivorans* are close to the OHR region (Figs 1 and 4), and phages have been reported to eventually play a role in horizontal gene transfer of reductive dehalogenases in *D. mccartyi* (McMurdie *et al.*, 2011; Waller *et al.*, 2012; Pöritz *et al.*, 2013). The large putative phage downstream of the OHR region (Fig. 1) includes genes coding for Tra-like proteins involved in pili-mediated conjugation (Anthony *et al.*, 1999). This finding supports a role of that region in horizontal gene transfer. Nineteen ORFs upstream of the *pceA* region genes coding for a SbcCD-like nuclease system (SMUL_1510-1511) are located. Such a nuclease system was reported to be involved in double strand breaking during DNA recombination (Connelly *et al.*, 1998).

A transposase is encoded by two genes located in close vicinity downstream of the corrinoid biosynthesis gene cluster. The insertion of the two transposase genes into a *tetR* transcriptional regulatory gene (Fig. 4) appears to be a stable genetic modification, as there are no flanking inverted repeats found. Another transposase gene is located 13 genes upstream of *pceA*. Several DNA sequence repeats were found in the OHR region employing REPUTER (Kurtz *et al.*, 2001) with a minimum repeat length of 10 bases, but the only inverted repeat was found upstream and downstream of the gene encoding for the alkylhydroperoxidase-like protein directly upstream of *pceA*. Other inverted or direct repeats were not found in the OHR region, arguing against the involvement of transposable elements during horizontal gene transfer via a transposon-based mechanism as discussed for *D. hafniense* strain TCE1 (Duret *et al.*, 2012).

Aerobic respiration and detoxification of reactive oxygen species

Sulfurospirillum multivorans was originally described as strictly anaerobic (Scholz-Muramatsu *et al.*, 1995), despite being a member of the ε-*Proteobacteria*, of which

most are nowadays described to be microaerophilic. To analyse the capability of *S. multivorans* to thrive in microaerobic environments, the growth of the organism under reduced oxygen concentrations was tested. With pyruvate as electron donor and carbon source, *S. multivorans* was able to grow in the presence of oxygen up to 15% in the gas phase (for details, see Fig. S5). With 5% oxygen in the gas phase as sole electron acceptor, the growth rate and cell density obtained was comparable with that of cultures grown with fumarate, PCE or nitrate as electron acceptors. The ability to respire oxygen is reflected in the gene inventory of the *S. multivorans* genome, where many genes are found, which are known to encode enzymes usually associated with an aerobic or microaerophilic lifestyle.

The genome of *S. multivorans* contains genes encoding key enzymes for detoxification of reactive oxygen species (ROS), namely catalase (SMUL_3224) and two superoxide dismutases (SODs) (SMUL_529 and SMUL_3084). While the first SOD is of the standard, iron-containing type found in most ε-*Proteobacteria* (Kern *et al.*, 2011a), the second one is of the Cu-Zn-type not encoded in other published ε-proteobacterial genomes and closely related to the SOD of γ-*Proteobacteria* (60% amino acid sequence identity). Genes for the catalase are rarely found in ε-proteobacterial genomes. It is present in the genomes of *S. barnesii*, *A. nitrofigilis*, *Arcobacter* strain L. and *Campylobacter showae*. Thus, the variety of genes encoding ROS-detoxifying enzymes in *S. multivorans* underlines the adaptation of this bacterium to oxic conditions.

Three different cytochrome *c* oxidases are found on the genome of *S. multivorans*. The cbb_3-type cytochrome *c* oxidase (SMUL_2651-2655) is present in all ε-proteobacterial genomes and is suggested to be responsible for the terminal reduction of oxygen in all ε-*Proteobacteria*, principally enabling them to thrive in microaerobic environments (Smith *et al.*, 2000). Another terminal oxidase encoded in the *S. multivorans* genome (SMUL_0740-0742) is an orthologue of an enzyme of *C. jejuni* recently described as a cyanide-insensitive oxidase of the *bd*-family that shows low affinity for O_2 (Jackson *et al.*, 2007; Borisov *et al.*, 2011). It seems to play a role in oxygen respiration and/or might be responsible for oxygen detoxification at higher O_2 concentrations. *Sulfurospirillum multivorans* is also coding for a third type of cytochrome oxidase (SMUL_920-923) similar to an enzyme encoded in *Desulfovibrio* spp. This protein was shown to be a cytochrome *c* oxidase of the cco/bo_3-type functional at higher oxygen concentrations (Ramel *et al.*, 2013). Apart from the occurrence of this type of cytochrome oxidase genes in *S. multivorans* and in the draft genome of *S. arcachonense*, it was not found in any other ε-proteobacterium.

The high number of cytochrome complexes in *S. multivorans* in comparison with other ε-*Proteobacteria* is unusual and might explain why this organism is able to cope with higher oxygen concentrations.

Respiration with other terminal electron acceptors

Sulfurospirillum multivorans is able to grow with a broad variety of terminal electron acceptors besides halogenated ethenes (Scholz-Muramatsu *et al.*, 1995). This is reflected in its genome because a wide spectrum of genes encoding terminal reductases is found. Genes coding for a typical fumarate reductase are present that resembles the protein investigated earlier in *W. succinogenes* (Lancaster *et al.*, 1999; Biel *et al.*, 2002). A gene encoding an MccA-type cytochrome *c* sulfite reductase (Kern *et al.*, 2011b) is present in the genome, although sulfite reduction of *S. multivorans* was never reported. Nitrate ammonification in *S. multivorans* is presumably achieved via the periplasmic nitrate and nitrite reductase system (Nap and Nrf, SMUL_934-940 and SMUL_889-892), which is described as responsible for nitrate ammonification in *W. succinogenes* (Simon, 2002; Kern and Simon, 2009). The nitrite reductase of *S. multivorans* is of the Nrf-type, described as a periplasmic polyheme-type enzyme mediating nitrite ammonification (Einsle *et al.*, 2000). The nitrate reductase is a molybdopterin-containing periplasmic enzyme of the Nap-type. A cytochrome *c* nitrous oxide reductase (cNosZ) similar to that of *W. succinogenes* (Simon *et al.*, 2004) is encoded in the *S. multivorans* genome (SMUL_2124), but it lacks the genes encoding the membrane-integral quinol dehydrogenase NosGH as well as the genes encoding proteins responsible for maturation of NosZ. Therefore, the *nosZ* gene product of *S. multivorans* might not be linked to a respiratory chain and might fulfil a different physiological function. The incomplete *nos* gene cluster, not found in any other *Sulfurospirillum* species so far, might have been horizontally transferred, as suggested by flanking transposase genes and an amino acid sequence identity of 80% to a *nosZ* gene product from *Sulfurimonas denitrificans*.

In the genome of *S. multivorans*, we found 20 different gene clusters encoding putative molybdopterin-containing oxidoreductases (Table 3) including the nitrate reductase (Nap) described above. Cultivation experiments of *S. multivorans* with trimethylamine N-oxide (TMAO), dimethyl sulfoxide (DMSO), polysulfide, perchlorate and tetrathionate as electron acceptors demonstrated that all these compounds support growth of *S. multivorans* (Figs S6–S8). These electron acceptors are often used as substrates for molybdopterin-containing reductases (Rothery *et al.*, 2008). Anaerobic

Table 3. Molybdopterin-containing oxidoreductases in *S. multivorans* and bidirectional BLAST hits in close relatives.

S. multivorans	*S. barnesii*	*S. deleyianum*	*W. succinogenes*	Product
SMUL_0079	Sulba_0053	Sdel_0039	WS0126	Fdh-H
SMUL_0272-0274	–	–	–	(DmsABC)
SMUL_0342-0344	Sulba_0281-0283	Sdel_0265-0267	WS0116-0118	Psr
SMUL_0346-0348	Sulba_0285-0287	Sdel_0269-0271	–	Thr
SMUL_0500-0501	–	Sdel_0361-0362	WS1849-1850	(TMAO/DMSO)
SMUL_0934-0937	Sulba_0795-0798	Sdel_0724-0727	WS1180-1177	Nap
SMUL_0950-0951	–	–	–	(MopB_3)
SMUL_0970-0972	Sulba_2214-2216	–	WS0027-0029 WS1146-1148	Fdh1
SMUL_1277-1278	–	–	–	(DMSOR)
SMUL_2141	Sulba_1550	Sdel_1503	–	(MopB_3)
SMUL_2312-2314	Sulba_0898-0900	–	WS1430-1432	(TMAO/DMSO)
SMUL_2568-2571	–	–	WS0936-0938	Ttr
SMUL_2871-2873	Sulba_0859-0862	Sdel_0805-0808	WS0733-0736	Fdh2
SMUL_2899-2901	Sulba_2201-2203	Sdel_2072-2075	–	Fdh3
SMUL_3029-3031	–	–	–	(Acet-Hydrat)
SMUL_3119-3120	Sulba_2413-2414	–	–	Aio
SMUL_3145-3147	Sulba_2418-2420	–	WS0763-0765	Arr
SMUL_3254-3256	–	–	–	(DMSORII)
SMUL_3273-3275	Sulba_2544-2546	Sdel_2273-2276	–	Psr/Sdh
SMUL_3280-3282	–	–	WS0707-0709	Psr/Sdh

Product row: putative encoded enzyme as revealed by BLASTp searches against the Swiss-Prot database combined with literature reports and search against the NCBI conserved domain database to retrieve the corresponding protein family (results from the latter in brackets).
Acet-Hydrat, acetate hydratase family protein; Aio, arsenite oxidase; Arr, arsenate reductase; DmsABC, trimeric DMSO reductase family; DMSOR, DMSO reductase family; DMSORII, DMSO reductase II family; Fdh1/2/3, periplasmic formate dehydrogenase; Fdh-H, cytoplasmic formate dehydrogenase; MopB_3, unidentified molybdopterin oxidoreductase family 3; Nap, periplasmic nitrate reductase; Psr, polysulfide reductase; Psr/Sdh, bidirectional polysulfide reductase/sulfide dehydrogenase; Thr, thiosulfate reductase; TMAO/DMSO, TMAO/DMSO reductase family; Ttr, tetrathionate reductase.

respiration of *S. multivorans* and other *Sulfurospirillum* spp. with arsenate and selenate, both substrates for molybdopterin-containing oxidoreductases (Schröder *et al.*, 1997; Malasarn *et al.*, 2004), was shown previously (Luijten *et al.*, 2004). Comparative genome-wide analysis showed that *S. multivorans* contains by far the highest number of gene clusters coding for molybdopterin oxidoreductases among ε-*Proteobacteria* (Table S1). Some of them could be assigned to a putative function according to amino acid sequence similarities to described enzymes (Table 3, 'product').

The amino acid sequences of the enzyme complex encoded by SMUL_0342-0344 show a high similarity (60–80% sequence identity) to the *W. succinogenes* polysulfide reductase (Krafft *et al.*, 1995), indicating an involvement of the encoded proteins in sulfur respiration. All *Sulfurospirillum* spp. are able to grow with polysulfide as terminal electron acceptor, and orthologues of the polysulfide reductase genes are present in all *Sulfurospirillum* spp. genome sequences. Two further putative molybdopterin oxidoreductases are encoded in *S. multivorans*, which might function as bidirectional polysulfide reductases/sulfide dehydrogenase, as shown for *W. succinogenes* (O. Klimmek, pers. comm.). To discriminate between DMSO and TMAO reductases is difficult because an ε-proteobacterial orthologue has not yet been described, and both enzymes exhibit high similarity. *Sulfurospirillum barnesii* does not utilize DMSO (Stolz *et al.*, 1999) whereas *S. deleyianum* grows with DMSO (Schumacher *et al.*, 1992) as electron acceptor. A likely candidate for DMSO reduction in *S. multivorans* is SMUL_0500-0501 because an orthologue is detectable in *S. deleyianum* rather than in the *S. barnesii* genome. TMAO as electron acceptor supported growth of *S. multivorans* and *S. barnesii*. Both organisms harbour an orthologous gene cluster, the products of which belong to the DMSO/TMAO reductase family (in *S. multivorans* SMUL_2312-2314).

The gene products of SMUL_2568-2571, a putative molybdopterin oxidoreductase, according to its amino acid sequence belongs to the tetrathionate reductase family, while the gene products of SMUL_346-348 are most likely responsible for thiosulfate respiration. The latter assumption is supported by the lack of an orthologue in the genome of *W. succinogenes*, which is not able to grow with thiosulfate, and by the reported growth of *S. deleyianum* (Schumacher *et al.*, 1992) and *S. barnesii* (Stolz *et al.*, 1999) with thiosulfate, both organisms harbouring the thiosulfate reductase orthologue. Arsenate respiration might be carried out by the *arrABC* gene products, which show a high amino acid sequence similarity to the described arsenate reductases (Malasarn *et al.*, 2004). Selenate as well as perchlorate respiration cannot be assigned to any gene product. The only gene

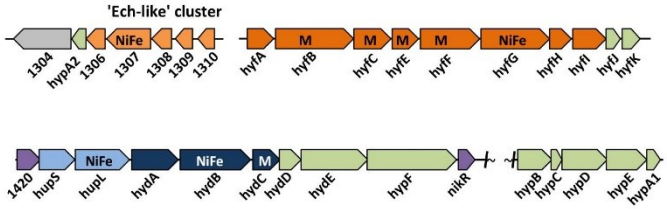

Fig. 5. Hydrogenase gene cluster of *S. multivorans*. Orange: genes encoding the Ech-like enzymes (light orange: 'Ech-like'; dark orange: membrane-bound Hyf-like hydrogenase); blue: putative hydrogen uptake hydrogenases (light blue: soluble, cytoplasmic hydrogenase); maturation genes shown in light green; regulatory genes in purple; genes with unknown functions shown in grey. Numbers correspond to the SMUL locus tags. 1304: putative pyridine dinucleotide binding oxidoreductase; NiFe: nickel-iron active site-containing subunit; M: membrane-spanning subunit.

product found in the *S. multivorans* genome bearing significant identity (> 30%) to already described enzymes carrying out these reductions is SMUL_3254-3256 with 35% and 30% amino acid identities of the large subunits respectively.

Electron-donating processes

Sulfurospirillum multivorans uses various organic and inorganic compounds as electron donors. Among them are H_2, H_2S, formate, pyruvate and lactate (Scholz-Muramatsu *et al.*, 1995). Three operons for putative selenocysteine-containing, periplasmic formate dehydrogenases (FDHs), each coding for a typical three-subunit FDH, were found in the genome sequence of *S. multivorans*. The catalytic subunits of these periplasmic FDHs have an identity of at least 60% to each other. One of the periplasmic FDHs was already described and the corresponding gene was sequenced (Schmitz and Diekert, 2003). This enzyme can be therefore assigned to the gene products of SMUL_2871-2873 and seems to be the main FDH as high activities of this enzyme were measured in formate-grown cells. Additionally, the corresponding operon is highly conserved in *S. barnesii* and *S. deleyianum*. The other two putative periplasmic FDHs (SMUL_2899-2901, SMUL_970-972) might play a role as backup or are differentially regulated, as is the case for *E. coli* (Sawers, 1994). In addition to the three periplasmic FDHs, a gene encoding a single-subunit cytoplasmic FDH (SMUL_0079), harbouring a cysteine-containing molybdopterin/tungstopterin binding motif, was detected. It is related to FDH H of *E. coli* (32% amino acid sequence identity). This enzyme might provide reducing equivalents for the anabolism when the organism is grown with formate and e.g. PCE. This physiological role has yet to be proven. The presence of four gene clusters encoding FDHs point towards a central role of formate in *S. multivorans*.

A putative periplasmic molybdopterin-containing arsenite oxidase is encoded by SMUL_3119-3120, consisting of two subunits and having a high amino acid identity (48%) to the corresponding enzyme found in *Alcaligenes faecalis* (Ellis *et al.*, 2001). Despite the presence of the arsenite oxidase gene cluster, attempts to grow *S. multivorans* in the presence of arsenite as electron donor (1–5 mM) and nitrate or oxygen as electron acceptor failed so far.

Lactate can be used as electron donor for growth of *S. multivorans* in the presence of most electron acceptors (Scholz-Muramatsu *et al.*, 1995). Lactate utilization is rare among ε-*Proteobacteria*, and *Sulfurospirillum* spp. are the only species carrying a gene coding for a NAD^+-dependent lactate dehydrogenase (SMUL_438 in *S. multivorans*). A lactate permease is encoded by SMUL_0863.

Hydrogen oxidation is widely distributed in ε-*Proteobacteria* and presumably carried out by a periplasmic membrane-bound NiFe hydrogenase (MBH) as described for *W. succinogenes* (Dross *et al.*, 1992). Genes encoding an MBH are present in *S. multivorans* (SMUL_1423-1425, Fig. 5), and this MBH is most likely responsible for hydrogen oxidation in this organism. The corresponding gene products are similar to the *W. succinogenes* enzyme and comprise a large subunit containing the active site (HydB), a small, electron-transferring subunit (HydA) and a membrane-integral cytochrome *b* (HydC). The electrons derived from H_2 oxidation are presumably transferred to the menaquinone pool via HydC. Three additional NiFe hydrogenase gene clusters are found in the genome of *S. multivorans* (Fig. 5). Located directly upstream of the MBH genes, two ORFs encoding a NiFe hydrogenase were detected (SMUL_1421-1422, *hupSL*, Fig. 5). Deduced from the amino acid sequence, these genes encode most likely a cytoplasmic uptake hydrogenase, belonging to the group 2 NiFe hydrogenases (Vignais and Billoud, 2007). A close relative is the hydrogenase III of *Aquifex aeolicus* (HupL: 36%, HupS: 43% amino acid sequence identity), where it might deliver low potential reducing equivalents for the reductive tricarboxylic acid (TCA) cycle (Guiral *et al.*,

2005). The role of this hydrogenase in *S. multivorans* is unclear. Despite the presence of genes for a reductive TCA cycle in the genome (see next chapter), autotrophic growth of *S. multivorans* could not be demonstrated yet. Another possible role of HupSL may be the recycling of cytoplasmic hydrogen originating from nitrogenase activity in *S. multivorans* (Ju *et al.*, 2007). This was described for cytoplasmic uptake hydrogenases of cyanobacteria (Tamagnini *et al.*, 2007), which are similar to HupSL of *S. multivorans*.

The two other gene clusters coding for NiFe hydrogenases have high amino acid sequence identities (ranging from 33% to 52%) to characterized group four hydrogenases of the Ech-type, i.e. energy-converting hydrogenases (Hedderich and Forzi, 2005). While one (SMUL_1306-1310, Fig. 5) has highest similarity to the CO-induced hydrogenase (Coo) of *Carboxydothermus hydrogenoformans* (Soboh *et al.*, 2002), the other is more related to hydrogenases 3 (Hyc) and 4 (Hyf) of *E. coli*, which are both supposed to have a function in the formate hydrogen lyase (FHL) complex (Bagramyan and Trchounian, 2003). Remarkably, the operon encoding the CO-induced hydrogenase-like enzyme of *S. multivorans* does not harbour genes encoding membrane proteins, which are normally found in Ech hydrogenases. Hence, there is no indication for the presence of a proton pump as proposed for other Ech hydrogenases. A gene coding for a CO dehydrogenase, normally found on genomes harbouring genes of a CO-induced hydrogenase, was not detected in the *S. multivorans* genome. Downstream of the structural genes for this Ech-like hydrogenase and a second *hypA* gene, a gene encoding a putative pyridine nucleotide-binding oxidoreductase is encoded (SMUL_1304, Fig. 5), the role of which is unclear. The Ech-like hydrogenase gene cluster including the oxidoreductase ORF is conserved in *S. barnesii* and *S. deleyianum*.

The fourth hydrogenase (Hyf) of *S. multivorans* resembles an FHL complex hydrogenase. It is encoded in an operon consisting of 10 genes (SMUL_2383-2392, Fig. 5). Eight of these seem to encode the structural subunits, similar to the *hyf* genes of *E. coli*. An FHL complex might be formed in *S. multivorans* because a gene encoding a cytoplasmic FDH is present. However, a physiological requirement for an FHL complex in *S. multivorans* is not obvious. Its role in *E. coli* is formate conversion to CO_2 and hydrogen when fermenting pyruvate by the pyruvate formate lyase complex (Knappe and Sawers, 1990). The latter complex is not encoded in the genome of *S. multivorans*.

Pyruvate is used as electron donor in *S. multivorans* (Scholz-Muramatsu *et al.*, 1995). *Sulfurospirillum multivorans* encodes two enzymes known to oxidize pyruvate. One is the pyruvate:ferredoxin oxidoreductase (PFOR, SMUL_2630), an enzyme often found in anaerobic and microaerobic microorganisms in place of a pyruvate dehydrogenase (Kerscher and Oesterhelt, 1982). The PFOR found in *S. multivorans* resembles the one found in *C. jejuni* (76% amino acid sequence identity). The second pyruvate-oxidizing enzyme is a putatively menaquinone-reducing pyruvate dehydrogenase (*poxB*, SMUL_1703), which is found in some facultative anaerobes such as *E. coli* (Grabau and Cronan, 1986). The role of the *poxB* gene product is not clear. This pyruvate dehydrogenase was described to support aerobic growth in *E. coli* (Abdel-Hamid *et al.*, 2001).

The electrons generated by pyruvate oxidation via PFOR are presumably transferred to ferredoxin or flavodoxin. In *Helicobacter pylori* and *C. jejuni*, it was shown that the electrons can be transferred to a $NADP^+$-specific flavodoxin:quinone reductase (FqrB) via a conserved flavodoxin. FqrB and the flavodoxin are conserved in ε-*Proteobacteria* (St Maurice *et al.*, 2007). *Sulfurospirillum multivorans* codes for both proteins as well (SMUL_1623 and SMUL_2785 respectively). FqrB then might reduce $NADP^+$ to NADPH, which can be used in anabolic reductions.

Central carbon metabolism, TCA cycle

Sulfurospirillum multivorans encodes genes for all enzymes participating in the reactions of the TCA cycle. The bacterium appears to use the ε-proteobacterial version of the TCA cycle for complete mineralization of organic compounds under (micro)aerobic conditions and for generation of reducing equivalents and building blocks for respiration and/or biosyntheses. For conversion of citrate, genes coding for three enzymes were found: aconitase (SMUL_1600), ATP citrate lyase (SMUL_0066-0067) and citrate (pro-3S)-lyase (SMUL_2346-2349). The aconitase mediates isocitrate formation, which is further oxidized to α-ketoglutarate by isocitrate dehydrogenase (SMUL_1442). The irreversible α-ketoglutarate dehydrogenase reaction using NAD^+ is replaced by the reversible α-ketoglutarate oxidation with ferredoxin (or flavodoxin) as electron acceptor mediated by the heterotetrameric α-ketoglutarate ferredoxin/flavodoxin oxidoreductase (SMUL_1452-1455).

A specific succinate dehydrogenase seems to be missing in ε-*Proteobacteria*. Instead, genes for a fumarate reductase (SMUL_0550-0552) similar to the one described for *W. succinogenes* are present in the genome of *S. multivorans*. This enzyme is characterized as functional in both directions, but primarily in the direction of fumarate reduction, and it is the key enzyme in fumarate respiration (Lancaster and Simon, 2002).

Two fumarate hydratases (fumarases) seem to be present in *S. multivorans*, similar to the *E. coli* enzymes

(Woods *et al.*, 1988). The class I fumarate hydratase (SMUL_1679-1680), in *E. coli* expressed under high concentrations of oxygen, is present also in several other free-living ε-*Proteobacteria*, but not in any of the host-associated species, as opposed to the class II fumarase (SMUL_1459), which is present in all ε-*Proteobacteria*. This might be another adaptation to higher oxygen concentrations.

Three genes encoding enzymes catalysing malate oxidation are present in the genome of *S. multivorans*, two pyridine nucleotide-dependent malate dehydrogenases (SMUL_0065 and 1443) and a membrane-bound malate:quinone oxidoreductase (MQO, SMUL_0667). The latter is also present in *H. pylori*, where it entirely replaces the malate dehydrogenase (Kather *et al.*, 2000). Malate dehydrogenase donates electrons to NAD^+, while MQO harbours FAD as cofactor and donates electrons to quinones, which are subsequently oxidized by components of the electron transfer chain.

Cytoplasmic electron carrier systems in S. multivorans

For the transfer of electrons generated in the cytoplasm by pyruvate oxidation or the TCA cycle, pyridine nucleotides (e.g. NADH), ferredoxins or flavodoxins may be used in *S. multivorans*. Six putative ferredoxins (apart from specific polyferredoxins) were found to be encoded in the genome of *S. multivorans*. Three 2[4Fe-4S]-type ferredoxins with a molecular weight of about 8–10 kDa (SMUL_0303, 0312 and 0908) are conserved in nearly all ε-*Proteobacteria* and may fulfill central roles in catabolism. Two of the other ferredoxins (SMUL_1217, another 2[4Fe-4S]-type ferredoxin, and SMUL_1235, a [2Fe2S]-type ferredoxin) are included, together with a flavodoxin (SMUL_1231), within the MoFe nitrogenase gene region. They might play a role in maturation of the MoFe nitrogenase or in providing electrons for N_2 fixation. The sixth ferredoxin (SMUL_3020) is clustered with a flavoprotein exhibiting similarities to fumarate reductases/succinate dehydrogenases and with three other flavoproteins of unknown function. Besides the nitrogenase flavodoxin, the only other flavodoxin in *S. multivorans* is encoded by SMUL_2785 (see above), presumably fulfilling central metabolic needs.

Gene clusters coding for two NADH:quinone oxidoreductase-like complexes were detected on the genome of *S. multivorans*. One of these (SMUL_195-208) is of the typical ε-proteobacterial type lacking the NADH binding subunits of complex I (NuoEF) that are replaced with distantly related homologues (Smith *et al.*, 2000). These distant homologues are also present in *C. jejuni* in which they have been shown to interact with flavodoxin (Weerakoon and Olson, 2008). Such an enzyme might link pyruvate oxidation upon ferredoxin or flavodoxin reduction to the electron transport chain. The second complex I is encoded by the gene cluster ranging from SMUL_508 to SMUL_521. The cluster is structured like the one from *E. coli* including *nuoEF* and with fused *nuoC* and *nuoD* genes. Such a NADH:quinone oxidoreductases is found in many *Sulfurospirillum* spp. including *S. barnesii*, *S. arcachonense* and *Sulfurospirillum* strain AM-N; however, it is absent in other ε-*Proteobacteria* sequenced so far. This enzyme may link the cytoplasmic reduction of NAD^+ mediated by different dehydrogenases to the electron transport chain in the cytoplasmic membrane. These two oxidoreductases may be another reason for the metabolic versatility of *Sulfurospirilllum* spp. with respect to the substrates that can be used as electron donors.

Nitrogen fixation

Besides using ammonium as nitrogen source, *S. multivorans* has been shown to fix molecular nitrogen (Ju *et al.*, 2007). The genome contains two clusters containing nitrogenase structural genes. One of these is of the molybdenum-iron type (SMUL_1286-1287), and the cluster contains all maturation genes required. The whole cluster seems to be widely distributed among the terrestrial ε-*Protobacteria*, while marine ε-*Proteobacteria* seem to lack the nitrogenase genes, revealed by a genome wide BLAST analysis of ε-proteobacterial genomes conducted in this study. An alternative, heterotrimeric putative Fe nitrogenase (related to the clostridial type) is not found in other ε-*Proteobacteria*. It is encoded in a cluster together with a flavoprotein of unknown function (SMUL_1656-1659). Growth experiments showed that *S. multivorans* can fix nitrogen in a medium not amended with molybdate (Fig. S9). *Sulfurospirillum barnesii*, harbouring the MoFe nitrogenase genes, but devoid of genes encoding a molybdenum-independent nitrogenase, was not able to grow with N_2 as sole nitrogen source without added molybdenum source, pointing towards the functionality of this type of nitrogenase in N_2 fixation by *S. multivorans*.

Conclusion

The elucidation of the genome sequence of *S. multivorans* provides insight into the versatile energy metabolism of this organism (Fig. 6) including organohalide respiration, which is clearly distinct from that of other organohalide-respiring bacteria. Genes encoding a putative quinol dehydrogenase were found which possibly play a role in transferring electrons from the quinone pool to the PceA. The downregulation of the corresponding genes after long-term cultivation without PCE supports this assumption. Furthermore, insights into the biosynthesis of the norpseudo-B_{12} cofactor of the PceA

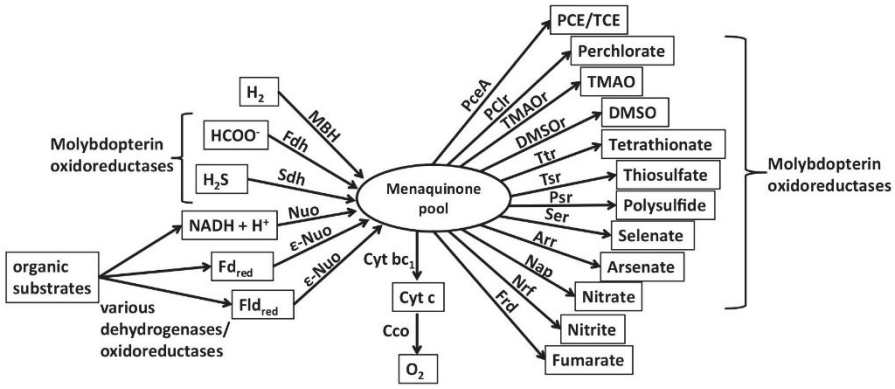

Fig. 6. Catabolic model of *S. multivorans* based on the physiological experiments and gene annotation of this study. Electron donors on the left side of the menaquinone pool, electron acceptors on the right side. Enzymes catalysing the oxidation of electron donors and the reduction of electron acceptors are given above each arrow. Arr: arsenate reductase; Cco: cytochrome c oxidase; Cyt: cytochrome; DMSOr: DMSO reductase; ε-Nuo: ferredoxin/flavodoxin-quinone oxidoreductase; Fdh: formate dehydrogenase; Frd: fumarate reductase; Nap: periplasmic nitrate reductase; Nrf: ammonifying nitrite reductase; Nuo: NADH-quinone oxidoreductase; PClr: perchlorate reductase; Psr: polysulfide reductase; Sdh: sulfide dehydrogenase; Ser: selenate reductase; TMAOr: TMAO reductase; Tsr: thiosulfate reductase; Ttr: tetrathionate reductase.

are given, pointing to a specific gene product involved in the formation of the unique linker moiety.

A high diversity of electron donor-oxidizing and electron acceptor-reducing enzymes was identified in *S. multivorans* based on a variety of oxidoreductases which was unseen before in ε-*Proteobacteria* (Fig. 6). This gene equipment can be especially useful for the organism to respire a broad spectrum of often hazardous and/or toxic compounds such as perchlorate, arsenate or selenate. Among the ε-*Proteobacteria*, *S. multivorans* is probably well able to deal with higher oxygen concentrations, as several terminal cytochrome c oxidases as well as different enzymes coping with reactive oxygen species are encoded in the genome. The physiological variability of the organism is also represented by the nitrogen assimilation gene inventory with two distinct nitrogenases. All in all, *S. multivorans* appears to be a highly versatile organism well equipped to survive and grow under many different environmental conditions.

Experimental procedures

Cultivation and DNA extraction

Sulfurospirillum multivorans was cultivated as described previously (Scholz-Muramatsu *et al.*, 1995). To test the substrate range of *S. multivorans*, a basal-defined medium as described (Scholz-Muramatsu *et al.*, 1995) was used with 40 mM pyruvate as electron donor and varying electron acceptors (oxygen, DMSO, TMAO, tetrathionate and perchlorate) or with PCE, nitrate (20 mM) or oxygen (5% gas in the headspace) as electron acceptor, and arsenite (1 mM or 5 mM) as electron donor. Yeast extract was omitted except where stated otherwise. Growth was recorded photometrically at 578 nm or, where optical density (OD) measurement was not applicable because of low growth or precipitation, via protein concentration measurement (Bradford, 1976). Isolation of genomic DNA was carried out by phenol-chloroform extraction (Bollet *et al.*, 1991) from cells grown with 40 mM pyruvate and 40 mM fumarate.

Genome sequencing and assembly

The genome of *S. multivorans* was sequenced using the 454 FLX sequencer with Titanium chemistry (Margulies *et al.*, 2005). A paired-end library with the average insert size of 8 kb was constructed, and 359 007 paired-end reads were generated. In total, the reads comprised 152 939 912 bp and represented an approximately 43-fold coverage of the genome. Newbler v. 2.3 (Roche) *de novo* assembly generated six scaffolds and 38 contigs longer than 2 kb. The contigs and scaffolds were oriented using MAUVE (Darling *et al.*, 2010) with the genome sequence of *S. deleyianum* (Sikorski *et al.*, 2010b) as a template, and the gaps between the contigs and scaffolds were closed by polymerase chain reaction, subsequent Sanger sequencing and primer walking.

Genome annotation and analysis of genetic elements

Initial annotation was performed using the RAST server (Aziz *et al.*, 2008), followed by a comparison with the annotated *S. deleyianum* and *S. barnesii* genomes and manual curation via the annotation platform ARTEMIS (Rutherford *et al.*, 2000). Manual curation involved a BLASTP search against the NCBI non-redundant (nr) and the SwissProt databases (Apweiler *et al.*, 2001) as well as motif searches via the NCBI conserved domains database CDD (Marchler-Bauer *et al.*, 2011) and INTERPROSCAN (Quevillon *et al.*, 2005). OriC was

determined using ORIFINDER (Gao and Zhang, 2008). The genome was searched for phages with PHAST (Zhou *et al.*, 2011) and PHISPY (Akhter *et al.*, 2012). *In silico* protein topology and transmembrane helix prediction analysis was performed with PHOBIUS (Käll *et al.*, 2004). The genome was searched for CRISPR regions using CRISPR FINDER with standard settings (Grissa *et al.*, 2007). Analysis of repeats was carried out with REPUTER without any distance values and a minimal repeat size of 8. The maximum of computed repeats was set to 100 to gather information about all repeats present. As template in REPUTER, the *S. multivorans* genome region starting at base 1 487 776 (upstream of the *rib* genes in front of the *pceA* cluster) and ending with base 1 531 179 (8 ORFs downstream of the corrinoid biosynthesis cluster) was used. The same sequence served as template in the search for regulatory genetic elements. Riboswitches and attenuators were investigated with RIBEX (Abreu-Goodger and Merino, 2005). For finding terminator structures, the tool ARNOLD (Naville *et al.*, 2011) was used; additionally, the OHR region sequence was scanned against the database WEBGESTER (Mitra *et al.*, 2011).

Comparative genome analyses

Whole genome BLAST was carried out with the BLAST+ standalone application (Camacho *et al.*, 2009) of NCBI (version 2.2.28) with standard settings against the refseq_protein database as downloaded from the NCBI server on 31 March 2014. Bidirectional BLAST analysis was carried out with BLAST+ and a cut-off threshold of an e-value of 10^{-6} and a sequence identity of 20%. The contigs of the draft genomes of *S. arcachonense* and of *Sulfurospirillum* strain AM-N were analysed via the available tools of the JGI server and additionally uploaded on the RAST server, where comparative BLAST analysis was performed via the sequence-based comparison tool.

Acknowledgements

This study was supported by grants from the German Research Foundation (DFG Research Unit FOR 1530). We are grateful for the excellent technical assistance of Peggy Brand-Schön in cultivation, DNA isolation and polymerase chain reaction execution. We would also like to thank Raffael Rubick for initial experiments on aerobic cultivation, Tom Petzold for initial growth experiments, Stefan Kruse for nitrogen fixation experiments, Marie Uksa for preliminary experiments on the second Rdh and Johannes Zimmermann for help with PHISPY. Marco Kai and Aleš Svatoš (Max Planck Institute for Chemical Ecology, Jena) are acknowledged for the mass spectrometric analysis of the isolated corrinoids, and Jörg Simon and Oliver Klimmek (TU Darmstadt) for helpful discussions on the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Reverse transcriptase PCR (RT-PCR) of genes coding for key enzymes of the OHR region (*pceA*: PCE reductive dehalogenase; *rdhA*: second putative reductive dehalogenase; SMUL_1541: putative quinol dehydrogenase periplasmic subunit; SMUL_1542: putative quinol dehydrogenase membrane subunit). The housekeeping gene *recA* was used as a control. DNA: genomic DNA as template in RT-PCR. +: RNA/cDNA was used as template. –: control with RNA but without reverse transcriptase. Py: pyruvate as electron donor; PCE: PCE as electron acceptor; Fu: fumarate as electron acceptor. HLII: Hyperladder II (Bioline, Germany). Total RNA of *S. multivorans* (1×109 cells) was isolated according to the manufactuerer's instructions using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The cells were disrupted by ultrasonic treatment (seven cycles of 2 s sonication and 2 s intermission, 25% amplitude; Bioblock Scientific Vibracell 75115, Illkirch, France). DNA contaminations were removed by incubating the RNA samples with DNase at 37°C for 1 h. The RT reaction was performed with 1 µg total RNA using the OneStep RT-PCR Kit (Qiagen) at 50°C for 1 h. The subsequent PCR was run as follows: initial PCR activation (15 min, 95°C) and 20 cycles of polymerization (1 min 94°C; 0,5 min 50°C; 1 min 72°C). The following primers (5′ → 3′) were used for the RT-PCR: *recA*: GGCGGTATTCCTCAAGGTCGTGTG and ATGTTCCGC ATCGACAAAGGCACAA (expected product size: 136 bp); *pceA*: AACACATTAAAAAATAAATAACTGTACTTGGGG and

TGAGTAAACGCTGTTCGTACTTCAGC (expected product size: 339 bp); *rdhA*: AACTAAGTTTTGGGTGTTTTCTATG and CTTTGTTATAGATATGTGGCCAATC (expected product size: 233 bp); *pceG*: GTTCGGTCTTGTTGGAGCTGTTC and CAATAAGTGTCACCGCTTTAGTGG (expected product size: 152 bp); *pceH*: TGTAAGAGGTTGGTC and CACATCTTTTACTTCTTT (expected product size: 277 bp).

Fig. S2. Phylogenetic tree of NapGH-like bacterial quinol dehydrogenases. The tree was generated with MEGA 6.0 (Tamura *et al.*, 2013) using the Muscle algorithm for alignment and maximum likelihood method (Felsenstein, 1981) for tree calculation. Amino acid sequences of the two subunits of the quinol dehydrogenases were concatenated. The bootstrap method with 1000 replications was used as phylogeny test. As the tree is not rooted, an outgroup was not used in the phylogenetic tree construction. Numbers shown are the corresponding bootstrap values, which show a very high confidence for each node, as 95% are generally considered to be statistically significant (Efron *et al.*, 1996). Accession numbers for amino acid sequences: *W. succinogenes* NapGH: NP_907362, NP_907361; *S. multivorans* NapGH: AHJ12202, AHJ12203; *E. coli* NapGH: NP_416709, NP_416708; *W. succinogenes* NosGH: NP_907123, NP_907127; *M. magneticum* NosGH: YP_422052, YP_422051; *S. multivorans* PceGH: AHJ12801, AHJ12802; *D. tiedjei* Desti_1408/1409: YP_006446386, YP_006446385.

Fig. S3. HPLC elution profile of the corrinoid isolated from *I. polytropus* compared with a standard mix of corrinoids. The corrinoid purification and analysis was conducted in accordance to Keller and colleagues (2013). The corrinoid standard mix contained norpseudovitamin B_{12} (Norps-B_{12}), norvitamin B_{12} (Nor-B_{12}), pseudovitamin B_{12} (Pseudo-B_{12}) and vitamin B_{12} (B_{12}). From the elution profile the production of pseudo-B_{12} in *I. polytropus* was deduced. Using LC-MS analysis [as described in Mac Nelly and colleagues (2014)], the respective corrinoid was identified as pseudovitamin B_{12} (the cyano-form of pseudo-B_{12}). Two ions were detected at m z^{-1} 1344.5410 $[M + H]^+$ and 672.7737 $[M + 2H]^{2+}$, which were in accordance to the monoisotopic masses of singly and doubly protonated pseudovitamin B_{12} with mass differences of 3.8 p.p.m. (calculated for $C_{59}H_{84}O_{14}N_{17}CoP$, 1344.5448) and 2.3 p.p.m. ($C_{59}H_{85}O_{14}N_{17}CoP$, 672.7760) respectively.

Fig. S4. Sequence alignment of CobD and histidinol-phosphate aminotransferase sequences. The alignment was generated with CLUSTAL OMEGA. CobD*Se* (acc. no. AAC79515; *Salmonella enterica*), CobD*Ip* (YP_003968483; *I. polytropus*), CobD*Sm* (AHJ12804; *S. multivorans*), HisC*Cj* (YP_002344819; *C. jejuni*), HisC*Sm* (AHJ11577; *Sulfurospirillum multivorans*), HisC*Ec* (NP_416525; *E. coli*). Residues highlighted in red in the CobD*Se* amino acid sequence were described to interact with L-threonine-*O*-3-phosphate or the pyridoxal cofactor (Cheong *et al.*, 2002).

Fig. S5. Growth curve of *S. multivorans* with oxygen as electron acceptor. Oxygen was supplied as 5% (squares), 10% (triangles), 15% (circles) or 20% (crosses) gas in headspace of 2 l gas-tight flasks with 200 ml anaerobized medium. Growth in medium without electron acceptor is marked with diamonds. The basal medium used is as described (Scholz-Muramatsu *et al.*, 1995) containing 0.2% yeast extract. Pyruvate (40 mM) was used as electron donor. The cultivation was performed at 28°C and 150 r.p.m. OD was measured photometrically (Pharmacia Biotech Novaspec II, Uppsala, Sweden).

Fig. S6. Growth curves of *S. multivorans* with either 10 mM perchlorate (squares), 10 mM chlorate (diamonds), 10 mM thiosulfate (circles) or without external electron acceptor (triangles). *Sulfurospirillum multivorans* (DSMZ 12446) was grown under anaerobic conditions at 28°C in a defined mineral medium (Scholz-Muramatsu *et al.*, 1995) without yeast extract and without vitamin B_{12} (cyanocobalamin). Pyruvate (40 mM) was used as electron donor. The cultivation of *S. multivorans* was performed in rubber-stoppered glass serum bottles. Protein was determined in accordance with the method described (Bradford, 1976) using the Bio-Rad reagent (Bio-Rad Laboratories, Munich, Germany). Bovine serum albumin was used as protein standard.

Fig. S7. Growth of *S. multivorans* with tetrathionate (triangles) and without external electron acceptor (circles). *Sulfurospirillum multivorans* was grown under anaerobic conditions at 28°C in a defined mineral medium (Scholz-Muramatsu *et al.*, 1995) without yeast extract. Pyruvate (40 mM) and tetrathionate (10 mM) was used as substrates. The cultivation was performed at 28°C and 150 r.p.m. OD was measured photometrically (Pharmacia Biotech Novaspec II, Uppsala, Sweden).

Fig. S8. Growth of different *Sulfurospirillum* spp. with DMSO (left) and TMAO (right) as electron acceptors. Squares: *S. deleyianum*; diamonds: *S. multivorans*; triangles: *S. barnesii*. The bacteria were grown on basal medium (Scholz-Muramatsu *et al.*, 1995) without yeast extract. 40 mM pyruvate and 20 mM DMSO or TMAO were used as substrates. The cultivation was performed at 28°C and 150 r.p.m. OD was measured photometrically (Pharmacia Biotech Novaspec II, Uppsala, Sweden).

Fig. S9. Growth of *S. multivorans* and *S. barnesii* with N_2 as sole nitrogen source. Molybdenum (0.35 μM final concentration of Na_2MoO_4) was omitted from the medium (left) or added to the medium (right). The bacteria were grown on basal medium (Scholz-Muramatsu *et al.*, 1995) without yeast extract and without NH_4Cl. 40 mM pyruvate and 40 mM fumarate were used as substrates. N_2 was added to the headspace (0.5 bar) in 200 ml serum bottles filled with 100 ml medium. The cultivation was performed at 28°C and 150 r.p.m. OD was measured photometrically (Pharmacia Biotech Novaspec II, Uppsala, Sweden).

Table S1. ε-Proteobacterial genomes with nine or more molybdopterin oxidoreductase genes. Based on a genome-wide BLAST analysis with the following different molybdopterin oxidoreductase large subunit amino acids sequences as queries: polysulfide reductase from *W. succinogenes* (acc. no. WP_011138081), formate dehydrogenase 1 from *S. multivorans* (acc. no. AHJ14110) and arsenate reductase from *Shewanella* sp. ANA-3 (acc. no. AAQ01672). The genomes from at least the type strain of ε-proteobacterial species were taken into account, as listed in the All Genomes Online Database 'GOLD' as of March 2014.

Supplemental material to:

Insights into organohalide respiration and the versatile catabolism of *Sulfurospirillum multivorans* gained from comparative genomics and physiological studies

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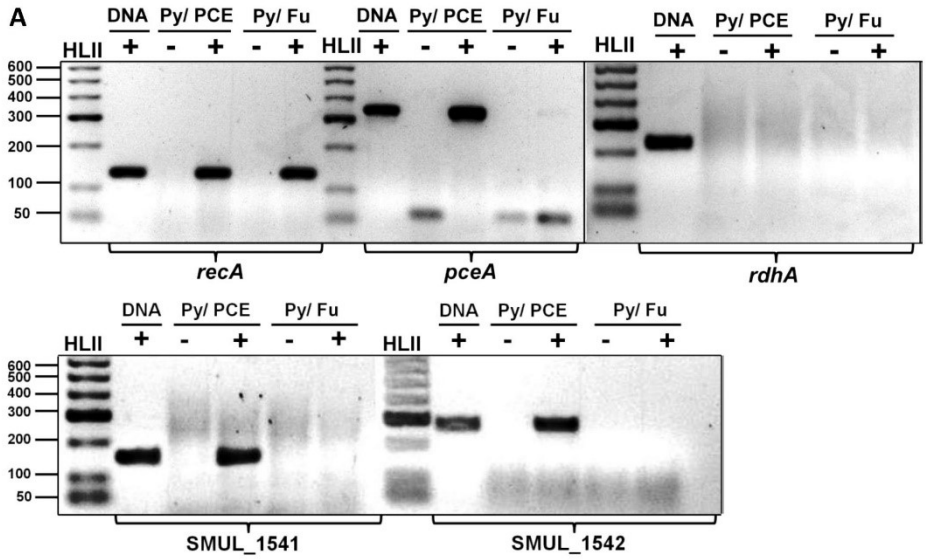

Figure S 1: Reverse transcriptase PCR of genes coding for key enzymes of the OHR region (*pceA*: PCE reductive dehalogenase, *rdhA*: second putative reductive dehalogenase, SMUL_1541: putative quinol dehydrogenase periplasmic subunit, SMUL_1542: putative quinol dehydrogenase membrane subunit). The housekeeping gene *recA* was used as a control. DNA: genomic DNA as template in RT-PCR. +: RNA/cDNA was used as template. -: control with RNA but without reverse Transcriptase. Py: Pyruvate as electron donor; PCE: PCE as electron acceptor; Fu: Fumarate as electron acceptor. HLII: Hyperladder II (bioline, Germany).
Total RNA of *S. multivorans* (1 x 10^9 cells) was isolated according to the manufactuerer's instructions using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The cells were disrupted by ultrasonic treatment (7 cycles of 2 sec sonication and 2 sec intermission, 25% amplitude; Bioblock Scientific Vibracell 75115, Illkirch France). DNA contaminations were removed by incubating the RNA samples with DNase at 37°C for 1 h. The reverse transcriptase (RT) reaction was performed with 1 µg total RNA using the OneStep RT-PCR Kit (Qiagen, Hilden, Germany) at 50°C for 1 h. The subsequent PCR was run as follows: initial PCR activation (15 min, 95°C) and 20 cycles of polymerization (1 min 94°C; 0,5 min 50°C; 1 min 72°C). The following primers (5' → 3') were used for the RT-PCR:
recA: GGCGGTATTCCTCAAGGTCGTGTG and ATGTTCCGCATCGACAAAGGCACAA (expected product size: 136 bp); *pceA*: AACACATTAAAAAATAAATAACTGTACTTGGGG and TGAGTAAACGCTGTTCGTACTTCAGC (expected product size: 339 bp); *rdhA*: AACTAAGTTTTGGGTGTTTTCTATG and CTTTGTTATAGATATGTGGCCAATC (expected product size: 233 bp); *pceG*: GTTCGGTCTTGTTGGAGCTGTTC and CAATAAGTGTCACCGCTTTAGTGG (expected product size: 152 bp); *pceH*: TGTAAGAGGTTGGTC and CACATCTTTTACTTCTTT (expected product size: 277 bp).

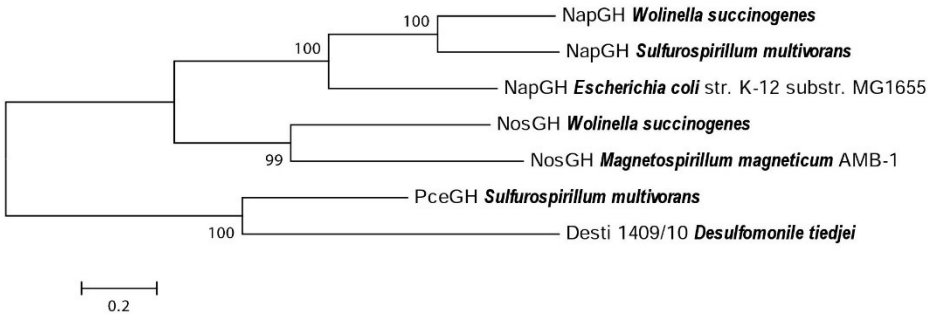

Figure S 2: Phylogenetic tree of NapGH-like bacterial quinol dehydrogenases. The tree was generated with Mega 6.0 (Tamura et al., 2013) using the Muscle algorithm for alignment and maximum likelihood method (Felsenstein, 1981) for tree calculation. Amino acid sequences of the two subunits of the quinol dehydrogenases were concatenated. The bootstrap method with 1000 replications was used as phylogeny test. As the tree is not rooted, an outgroup was not used in the phylogenetic tree construction. Numbers shown are the corresponding bootstrap values, which show a very high confidence for each node, as 95% are generally considered to be statistically significant (Efron et al., 1996). Accession numbers for amino acid sequences: *W. succinogenes* NapGH: NP_907362, NP_907361; *S. multivorans* NapGH: AHJ12202, AHJ12203; *E. coli* NapGH: NP_416709, NP_416708; *W. succinogenes* NosGH: NP_907123, NP_907127; *M. magneticum* NosGH: YP_422052, YP_422051; *S. multivorans* PceGH: AHJ12801, AHJ12802; *D. tiedjei* Desti_1408/1409: YP_006446386, YP_006446385

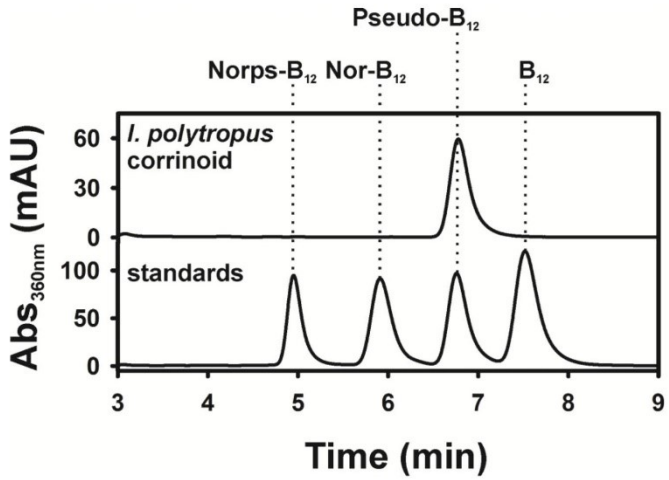

Figure S 3: HPLC-elution profile of the corrinoid isolated from *Ilyobacter polytropus* compared to a standard mix of corrinoids. The corrinoid purification and analysis was conducted in accordance to Keller et al. (2013). The corrinoid standard mix contained norpseudovitamin B_{12} (Norps-B_{12}), norvitamin B_{12} (Nor-B_{12}), pseudovitamin B_{12} (Pseudo-B_{12}), and vitamin B_{12} (B_{12}). From the elution profile the production of pseudo-B_{12} in *I. polytropus* was deduced. Using LC-MS analysis (as described in Mac Nelly et al. (2014)) the respective corrinoid was identified as pseudovitamin B_{12} (the cyano-form of pseudo-B_{12}). Two ions were detected at m/z 1,344.5410 $[M+H]^+$ and 672.7737 $[M+2H]^{2+}$, which were in accordance to the monoisotopic masses of singly and doubly protonated pseudovitamin B_{12} with mass differences of 3.8 ppm (calculated for $C_{59}H_{84}O_{14}N_{17}CoP$, 1,344.5448) and 2.3 ppm ($C_{59}H_{85}O_{14}N_{17}CoP$, 672.7760), respectively.

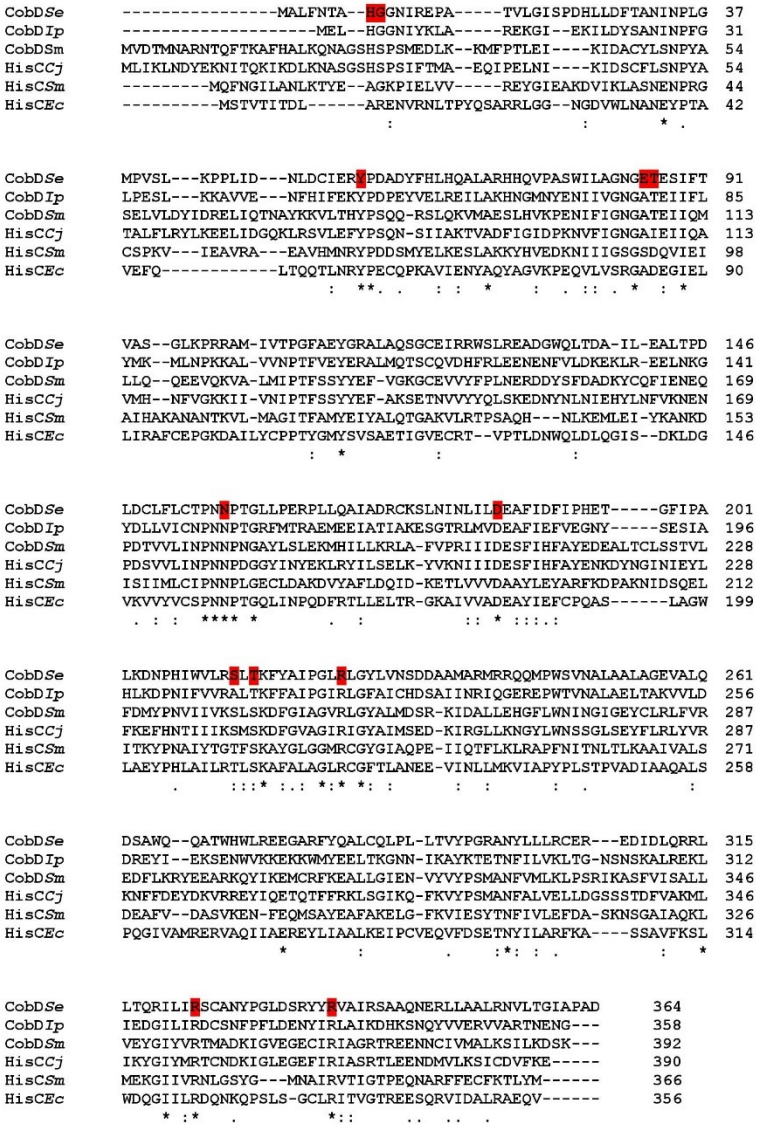

Figure S 4: Sequence alignment of CobD and histidinol-phosphate aminotransferase sequences. The alignment was generated with Clustal Omega. CobD*Se* (acc. no. AAC79515; *Salmonella enterica*), CobD*Ip* (YP_003968483; *Ilyobacter polytropus*), CobD*Sm* (AHJ12804; *Sulfurospirillum multivorans*), HisC*Cj* (YP_002344819; *Campylobacter jejuni*), HisC*Sm* (AHJ11577; *Sulfurospirillum multivorans*), HisC*Ec* (NP_416525; *Escherichia coli*). Residues highlighted in red in the CobD*Se* amino acid sequence were described to interact with L-threonine-*O*-3-phosphate or the pyridoxal cofactor (Cheong et al., 2002).

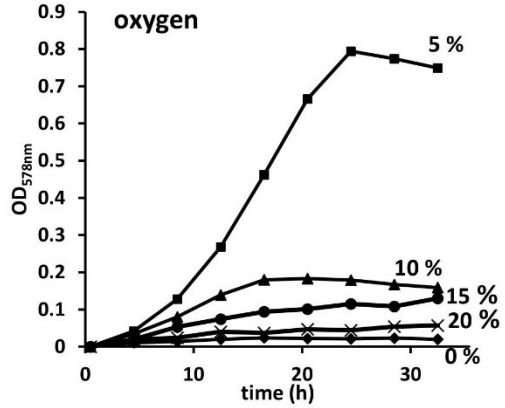

Figure S 5: Growth curve of *S. multivorans* with oxygen as electron acceptor. Oxygen was supplied as 5% (squares), 10% (triangles) 15% (circles) or 20% (crosses) gas in headspace of 2L gas-tight flasks with 200ml anaerobized medium. Growth in medium without electron acceptor is marked with diamonds. The basal medium used is as described (Scholz-Muramatsu et al., 1995) containing 0.2% yeast extract. Pyruvate (40 mM) was used as electron donor. The cultivation was performed at 28°C and 150 rpm. Optical density (OD) was measured photometrically (Pharmacia Biotech Novaspec II, Uppsala, Schweden).

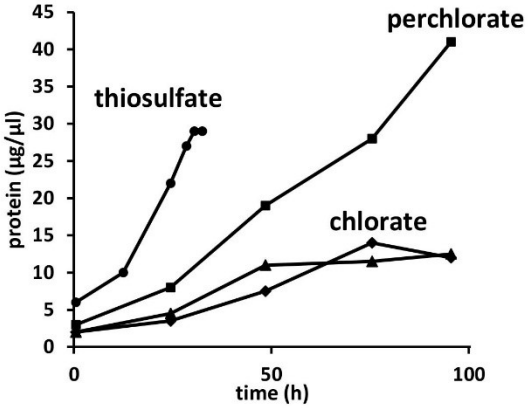

Figure S 6: Growth curves of *S. multivorans* with either 10mM perchlorate (squares), 10mM chlorate (diamonds), 10mM thiosulfate (circles) or without external electron acceptor (triangles). *S. multivorans* (DSMZ 12446) was grown under anaerobic conditions at 28°C in a defined mineral medium (Scholz-Muramatsu et al., 1995) without yeast extract and without vitamin B_{12} (cyanocobalamin). Pyruvate (40 mM) was used as electron donor. The cultivation of *S. multivorans* was performed in rubber-stoppered glass serum bottles. Protein was determined in accordance with the method described (Bradford, 1976) using the Bio-Rad reagent (Bio-Rad Laboratories, Munich, Germany). Bovine serum albumin was used as protein standard.

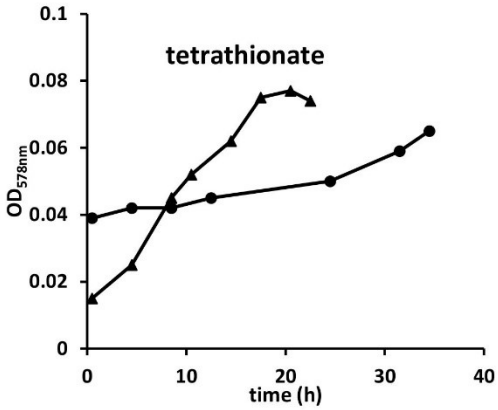

Figure S 7: Growth of *S. multivorans* with tetrathionate (triangles) and without external electron acceptor (circles). *S. multivorans* was grown under anaerobic conditions at 28°C in a defined mineral medium (Scholz-Muramatsu et al., 1995) without yeast extract. Pyruvate (40 mM) and tetrathionate (10 mM) was used as substrates. The cultivation was performed at 28°C and 150 rpm. Optical density (OD) was measured photometrically (Pharmacia Biotech Novaspec II, Uppsala, Schweden).

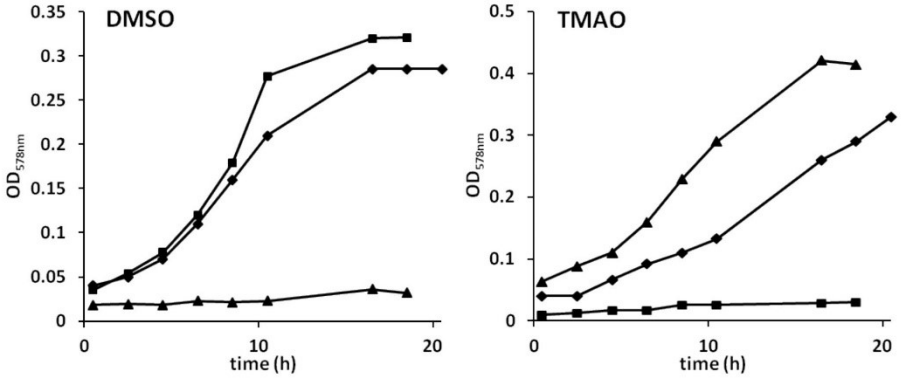

Figure S 8 Growth of different *Sulfurospirillum* spp. with DMSO (left) and TMAO (right) as electron acceptors. Squares: *S. deleyianum*, diamonds: *S. multivorans*, triangles: *S. barnesii*. The bacteria were grown on basal medium (Scholz-Muramatsu et al., 1995) without yeast extract. 40 mM pyruvate and 20 mM DMSO or TMAO were used as substrates. The cultivation was performed at 28°C and 150 rpm. Optical density (OD) was measured photometrically (Pharmacia Biotech Novaspec II, Uppsala, Schweden).

Table S 1: ε-proteobacterial genomes with nine or more molybdopterin oxidoreductase genes. Based on a genome-wide blast analysis with the following different molybdopterin oxidoreductase large subunit amino acids sequences as queries: Polysulfide reductase from *W. succinogenes* (acc. no. WP_011138081), formate dehydrogenase 1 from *S. multivorans* (acc. no. AHJ14110), arsenate reductase from *Shewanella* sp. ANA-3 (acc. no. AAQ01672). The genomes from at least the type strain of ε-proteobacterial species were taken into account, as listed in the All Genomes Online Database "GOLD" as of March 2014.

Species	molybdopterin oxidoreductase genes
Sulfurospirillum multivorans	20
Sulfurospirillum strain Am-N (draft)	11
Sulfurospirillum arcachonense (draft)	10
Sulfurospirillum barnesii	12
Sulfurospirillum deleyianum	10
Wolinella succinogenes	11
Arcobacter nitrofigilis	9
Arcobacter strain CAB (draft)	11

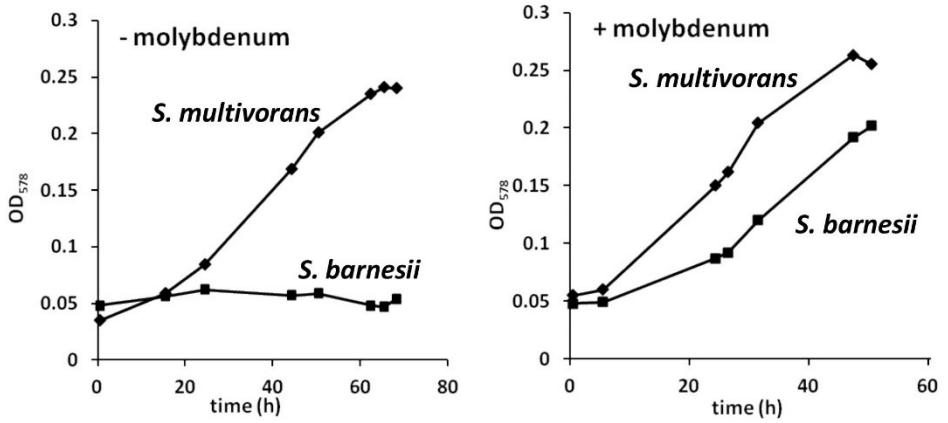

Figure S 9 Growth of *S. multivorans* and *S. barnesii* with N_2 as sole nitrogen source. Molybdenum (0.35µM final concentration of Na_2MoO_4) was omitted from the medium (left) or added to the medium (right). The bacteria were grown on basal medium (Scholz-Muramatsu et al., 1995) without yeast extract and without NH_4Cl. 40 mM pyruvate and 40 mM fumarate were used as substrates. N_2 was added to the headspace (0.5 bar) in 200 ml serum bottles filled with 100 ml medium. The cultivation was performed at 28°C and 150 rpm. Optical density (OD) was measured photometrically (Pharmacia Biotech Novaspec II, Uppsala, Schweden).

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Efron, B., Halloran, E., and Holmes, S. (1996) Bootstrap confidence levels for phylogenetic trees. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 7085-7090.
Felsenstein, J. (1981) Evolutionary trees from DNA-sequences: A maximum-likelihood approach. *Journal of Molecular Evolution* **17**: 368-376.
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Mac Nelly, A., Kai, M., Svatoš, A., Diekert, G., and Schubert, T. (2014) Functional heterologous production of reductive dehalogenases from *Desulfitobacterium hafniense* strains. *Appl Environ Microbiol*.
Scholz-Muramatsu, H., Neumann, A., Messmer, M., Moore, E., and Diekert, G. (1995) Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. *Archives of Microbiology* **163**: 48-56.
Sievers, F., and Higgins, D.G. (2014) Clustal Omega, accurate alignment of very large numbers of sequences. *Methods Mol Biol* **1079**: 105-116.
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3.2 Manuskript II

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SCIENTIFIC REPORTS

OPEN

Received: 25 February 2015
Accepted: 05 August 2015
Published: 21 September 2015

Proteomics of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates

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Organohalide respiration is an environmentally important but poorly characterized type of anaerobic respiration. We compared the global proteome of the versatile organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* grown with different electron acceptors (fumarate, nitrate, or tetrachloroethene [PCE]). The most significant differences in protein abundance were found for gene products of the organohalide respiration region. This genomic region encodes the corrinoid and FeS cluster containing PCE reductive dehalogenase PceA and other proteins putatively involved in PCE metabolism such as those involved in corrinoid biosynthesis. The latter gene products as well as PceA and a putative quinol dehydrogenase were almost exclusively detected in cells grown with PCE. This finding suggests an electron flow from the electron donor such as formate or pyruvate via the quinone pool and a quinol dehydrogenase to PceA and the terminal electron acceptor PCE. Two putative accessory proteins, an IscU-like protein and a peroxidase-like protein, were detected with PCE only and might be involved in PceA maturation. The proteome of cells grown with pyruvate instead of formate as electron donor indicates a route of electrons from reduced ferredoxin via an Epsilonproteobacterial complex I and the quinone pool to PCE.

Halogenated hydrocarbons of anthropogenic origin have been widely used, e.g. as solvents or pesticides, during the last two centuries and are among the major pollutants in the environment. Additionally, organohalides are formed in biogeochemical processes and are therefore abundant in nature since billions of years. As a consequence, it is not surprising that microorganisms have adapted to exploit these compounds as nutrients in the course of the evolution[1]. Under anoxic conditions, halogenated hydrocarbons may be dehalogenated by reductive dehalogenation that is performed by bacteria and can be coupled to ATP synthesis via electron transport phosphorylation (organohalide respiration). Organohalide-respiring bacteria (OHRB) are found in phylogenetically diverse bacterial phyla like the *Proteobacteria*, *Firmicutes* and *Chloroflexi*[2,3] and are classified as obligate or non-obligate organohalide respirers.

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The Epsilonproteobacterium *Sulfurospirillum multivorans* (formerly *Dehalospirillum multivorans*) was isolated from activated sludge of a waste water treatment plant[4]. *S. multivorans* is a non-obligate OHRB, which reductively dehalogenates tetrachloroethene (PCE) and trichloroethene (TCE) to *cis*-1,2-dichloroethene (*c*DCE)[5]. The organism is able to utilize several different electron donors (e.g. hydrogen, formate, pyruvate, lactate) and a broad range of electron acceptors (e.g. fumarate, nitrate and many others besides PCE) for anaerobic respiration[4,6]. The key enzyme of PCE respiration is the PCE reductive dehalogenase (PceA), a corrinoid and FeS cluster containing enzyme, which is attached to the periplasmic face of the cytoplasmic membrane most probably via the putative membrane-anchor protein PceB[7,8]. The genome of *S. multivorans* is described and contains 3,233 protein-coding sequences[6]. Among them, a 50 kbp gene region was identified, which is not present in non-organohalide respiring *Sulfurospirillum* species. This region contains, among others, the *pceAB* operon, a second reductive dehalogenase gene cluster, genes for two-component regulatory systems, a set of genes encoding proteins involved in corrinoid biosynthesis and several genes encoding for proteins with putative accessory functions[6]. The corrinoid cofactor of PceA, a norpseudo-B_{12}[9], is synthesized *de novo* in *S. multivorans*[10]. This special type of corrinoid cofactor is not known to be produced by any other bacterium. Unlike in other OHRB, two genes encoding a putative quinol dehydrogenase were identified adjacent to the reductive dehalogenase structural genes[6]. The corresponding gene products show similarities to the NapGH quinol dehydrogenase of *Wolinella succinogenes*, which transfers electrons from the menaquinone pool to the terminal reductase in nitrate respiration, the periplasmic NapA[11]. In the absence of PCE as terminal electron acceptor during several transfers of *S. multivorans*, the *pceA* expression gradually ceased[12]. Based on this result, the generation of *S. multivorans* cells lacking PceA is possible. Recently, the quinol dehydrogenase genes in close proximity to the *pce* gene cluster were shown to undergo a similar transcriptional down-regulation in the absence of PCE[6].

The PCE-dependent transcriptional up-regulation of the mentioned genes raised the need to validate these findings on the protein level and to compile global proteomic profiles specific for different electron donors and acceptors. The versatile metabolic capacities of *S. multivorans* allowed for a comprehensive comparison of proteome profiles originating from cultivations with pyruvate or formate as electron donors and either PCE, fumarate or nitrate as electron acceptors. This analysis allows for conclusions on the protein inventory involved in the PCE respiratory chain and in maturation of the required cofactors and proteins.

Results

Protein identification and relative quantification. Diverse substrate combinations were applied to analyze the proteome profile of *S. multivorans*. Pyruvate and fumarate (Py/Fu) was used as the standard condition with the following electron donor/acceptor combinations for comparison: pyruvate with nitrate (Py/Ni) or PCE (Py/PCE); formate with fumarate (Fo/Fu), PCE (Fo/PCE) or nitrate (Fo/Ni). After harvest and disruption of cells, samples were fractionated into soluble fraction (SF) and membrane extract (ME). Subsequently, the proteomes of SF and ME were analyzed by LC-MS (Fig. 1). Between 689 and 918 proteins were identified in a single LC-run. In total, 1,716 distinct proteins were identified under at least one cultivation condition (Table S1), which results in 53% coverage of the annotated 3,191 non-redundant protein-coding sequences of *S. multivorans*. Among all conditions, we were able to quantify 616 proteins. Of these, 241 proteins were predicted to contain between one and 15 putative transmembrane helices calculated by an *in silico* topology analysis with Phobius[13]. The counts for those putative membrane proteins were from twofold (Py/Ni) to fivefold (Fo/Ni) higher in the membrane extract compared to the soluble fractions.

A principal component analysis (PCA) of quantified proteins was performed as indicator of the variance in the dataset. We observed that the biological replicates cluster appropriately, membrane extract and the soluble fractions can be distinguished and the dataset revealed reasonable reproducibility (Fig. 2).

Overview on the PCE-induced protein pattern. Proteome analysis of *S. multivorans* grown with PCE, fumarate or nitrate as electron acceptors led to the identification of 119 proteins exclusively present in PCE-grown cells (Table S2). From these 119 proteins, 25 were quantified reliably (i.e. quantified in at least 50% of the biological replicates). Another 16 proteins were quantified in all samples with at least 2-fold higher protein abundance in Py/PCE and Fo/PCE cells compared to nitrate or fumarate (Fig. S1 and Table S3). The protein abundance of 28 proteins was significantly lower (at least 3-fold, $p < 0.05$ or not identified, Table S3) in Py/PCE and Fo/PCE cells when compared to the corresponding fumarate- or nitrate-grown cells.

Proteins of the OHR region. Previously, an approximately 50 kbp large region was identified in the genome of *S. multivorans* with no similar genes found in closely related *Sulfurospirillum* spp. genomes, encoding proteins directly (for example PceA) or indirectly (e.g. for the corrinoid cofactor biosynthesis) involved in organohalide respiration. This region, ranging from SMUL_1516 to SMUL_1596, was therefore termed OHR (organohalide respiration) region[6]. Of the proteins encoded in the OHR region, 46 have a functional correlation to organohalide respiration and are located in the OHR "core" region (SMUL_1530 to SMUL_1575). Of them, 29 were identified and 24 were quantified with PCE as electron acceptor in at least one of the four samples (Py/PCE-ME, Py/PCE-SF, Fo/PCE-ME, Fo/PCE-SF; Fig. 3).

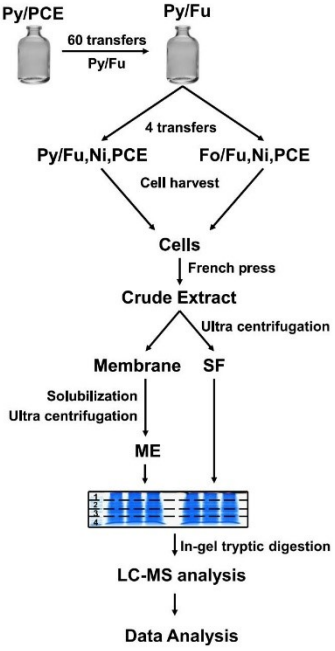

Figure 1. Experimental design of the differential proteomics approach used in this study. Grown cultures of *S. multivorans* were harvested and prepared for LC-MS measurement after repeated transfers (10% inoculum) on the desired substrates (Py, pyruvate; Fo, formate; Fu, fumarate; Ni, nitrate; PCE, tetrachloroethene) to get rid of any residual amounts of previously applied electron acceptors. The initial 60 transfers on Py/Fu were carried out to achieve down-regulation of PCE respiration in *S. multivorans* cells[12]. Membrane protein extract (ME) and soluble protein fraction (SF) were subjected to 1D-SDS PAGE. After in-gel digestion, peptide lysates were analyzed by LC-MS.

Products of the genes which are located upstream or downstream of this OHR "core" region were not detected. In cells grown without PCE as electron acceptor, 14 of the 46 proteins of the "core" OHR region were detected in at least one of the eight samples. Only four of these 14 proteins could be quantified. Of these, the genes encoding PceA (SMUL_1531), CbiC (SMUL_1555) and a putative FMN-binding protein (SMUL_1575), were detected in significantly higher levels in PCE-grown cells (at least 50-times higher with $p < 0.05$ or not present without PCE; CbiC with $p = 0.06$ in Py/PCE-SF vs PyNi-SF, Fig. 3, Table S3) while a two-component response regulator (SMUL_1539) was not differently produced. PceA is subjected to a long term regulation[12] and the other proteins encoded in the OHR core region follow presumably a similar pattern, with no or very low amounts detected in long-term cultivated cells without PCE (Fig. 3).

The PCE reductive dehalogenase PceA, the key enzyme of the PCE respiratory chain (Fig. 4), represents the most abundant protein encoded on the OHR region (Fig. 3) and is in general among the most abundant proteins in *S. multivorans* cells grown with PCE as electron acceptor (Table S4). In PCE-grown cells, PceA was present in an at least 32-fold higher amount (see Table S3). These data are in accordance to immunoblot analysis of the PceA protein and PceA activity of cell extracts measured in the same samples (Fig. S2, Table S4). PceB, the putative membrane anchor of PceA[8], predicted to contain two transmembrane helices, was found exclusively in Py/PCE-ME and Fo/PCE-ME (Fig. 3). The second reductive dehalogenase (Fig. 3) was identified at low levels in Fo/PCE-cells only, while RdhB, the corresponding putative membrane anchor was not detected. Downstream of the second *rdhA* cluster, two genes are located which encode a putative quinol dehydrogenase (Fig. 3). One of the corresponding products, the putative periplasmic FeS cluster containing subunit (SMUL_1541) could be quantified exclusively in the membrane extracts of Fo/PCE and Py/PCE-grown cells. The membrane-integral subunit of the quinol dehydrogenase (SMUL_1542) was not detected, which might be attributed to its tight interaction with the membrane.

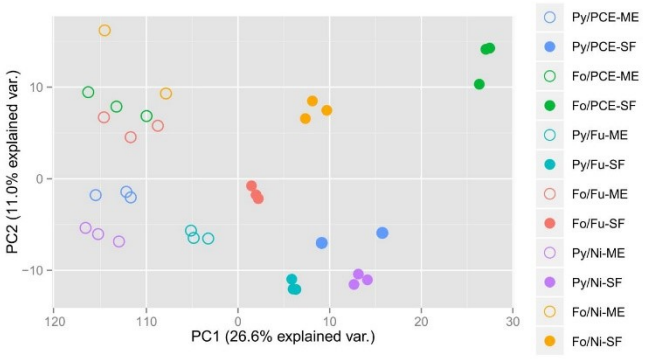

Figure 2. Principal component analysis of proteome profiles. Cultivation conditions are indicated by different colors, membrane samples are marked with open circles, soluble fractions by filled circles. The first two letters represent the electron donor: Fo, formate; Py, pyruvate, followed by the electron acceptor Fu, fumarate; Ni, Nitrate and fraction ME, membrane extract; SF, soluble fraction. Proteins quantfied in at least half of all measurements were included in the analysis (n = 672). The conditions Fo/Ni-ME and Py/PCE-SF include two points only, as one of the biological triplicates of each were identified as outliers using statistical analysis described in the methods section and were therefore not considered in the data analysis.

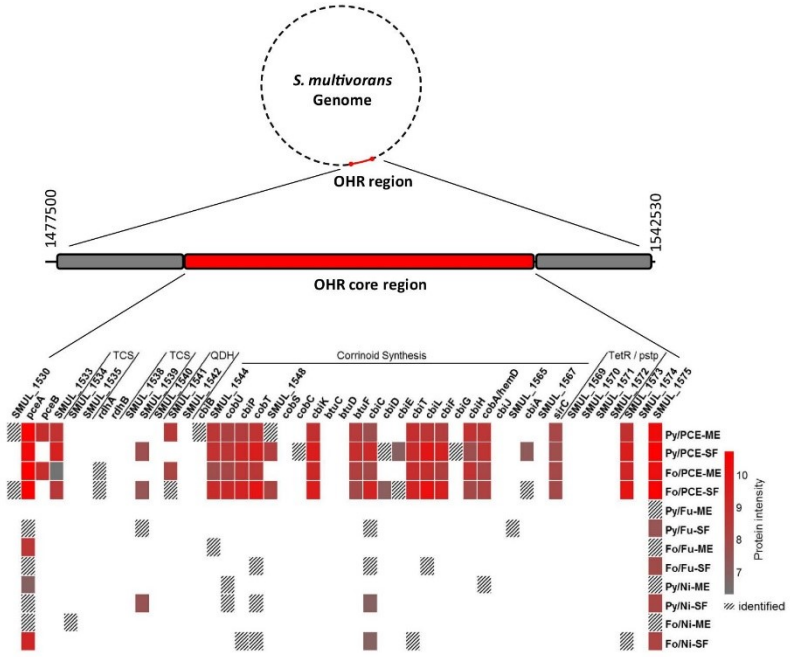

Figure 3. Identified gene products of the OHR core region as detected by proteomic analyses. Gene names or locus tags are given at the top of the protein intensity pattern. Each square correlates to a given gene product identified or quantified under a given cultivation condition (at the right). For quantified proteins the protein intensity is provided (color code at the far right, normalized and logarithmized average of top 3 peptide area as described in the methods section), proteins identified but not quantified are marked with shaded squares. Genes which functionally belong together are grouped by flanking lines and given the following abbreviations: TCS, two component regulator system; QDH, quinol dehydrogenase; TetR/pstp, *tetR* pseudogene disrupted by transposase.

The following proteins encoded in the OHR region were exclusively identified in cells cultivated with PCE as electron acceptor. Directly upstream of *pceA*, an alkylhydroperoxidase-like-protein is encoded by SMUL_1530. This protein was identified in Py/PCE-ME and Fo/PCE-SF only. A small protein with unknown function (SMUL_1533) is encoded downstream of the *pceAB* gene cluster and was quantified in all PCE-samples. It shows low similarities to proteins involved in FeS cluster maturation (21% amino acid sequence identity to the characterized *E. coli* IscU[14]). SMUL_1533 was quantified in all PCE-samples. It showed protein values (normalized and logarithmized average of top 3 peptide area) of 9.4 in Py/PCE-SF and 8.5 in Fo/PCE-SF, the 3 to 10-fold compared to the membrane extracts (Fig. 3). The amount of IscSU/NifSU (SMUL_2994-2995), the epsilonproteobacterial FeS cluster biosynthetis proteins[15] is not significantly altered in the presence of PCE. A small putative membrane protein (SMUL_1540) with three predicted transmembrane helices was not identified. Downstream of each reductive dehalogenase gene cluster, a two-component regulator system is encoded. Each of them includes a putative histidine-protein kinase (HPK; SMUL_1534 and SMUL_1538) and a putative response regulator (RR; SMUL_1535 and SMUL_1539). The HPK SMUL_1534 was identified only in Fo/Ni-ME, while the HPK protein SMUL_1538 was not detected. The RR protein SMUL_1539 was identified in Py/Fu-SF and quantified (7.4 to 7.6) in Py/PCE-SF, Py/Ni-SF and Fo/PCE-SF (Fig. 3). The RR protein SMUL_1535 could not be identified.

Corrinoid biosynthesis. PceA harbors a corrinoid cofactor shown to be essential for the dehalogenation process[16]. The corrinoid cofactor of PceA is a norpseudo-B_{12}[9] and synthesized *de novo* by *S. multivorans*[10]. The genes encoding the proteins necessary for corrinoid biosynthesis are located in the OHR region downstream of the reductive dehalogenase gene clusters and the quinol dehydrogenase genes[6]. All proteins which are expected to be part of the corrinoid biosynthesis machinery were identified and quantified in the proteome of *S. multivorans*, with the exception of CobS (cobalamin 5′-phosphate synthase, SMUL_1549) and CbiJ (cobalt-precorrin-6x reductase, SMUL_1564). CobS is predicted to be membrane-integral through seven transmembrane helices, which might be the reason for the apparent absence of the protein in the proteome. CbiB (adenosylcobinamide-phosphate synthase, SMUL_1543), which is predicted to contain six transmembrane helices, was identified only in Py/PCE-ME. Almost all proteins of the corrinoid biosynthesis identified in this study were exclusively detected in PCE-grown cells or their levels were at least significantly higher in the presence of PCE (Fig. 3, Tables S2 and S3). Seventeen of the 20 identified proteins of the corrinoid biosynthesis cluster could be quantified in at least one sample of PCE-grown cells. The proteins CobC (alpha-ribazole phosphatase, SMUL_1550) and CbiG (cobalamin biosynthesis protein, SMUL_1561) could be identified only in Py/PCE-SF. In general, proteins detected in a lower amount or identified once or not at all, are involved in corrin ring biosynthesis and modification (CbiD, CbiE, CbiA, CbiG, CbiJ). A unique enzyme putatively involved in *de novo* corrinoid biosynthesis of *S. multivorans* is SMUL_1544, which displays very low sequence identity to biochemically characterized threonine phosphate decarboxylases (CobD)[6]. This protein might be responsible for the production of ethanolamine phosphate, which may be incorporated as linker moiety into the norpseudo-B_{12}'s nucleotide loop. It was detected in the soluble fractions of all PCE-grown cells in a significant amount (8.8 to 9.0) at 2 to 5-times higher levels than in the membrane extracts. Of the corrinoid ABC transporter BtuCDF, only the periplasmic component BtuF was detected. Neither BtuC nor BtuD was detected, which might be due to their tight interaction with the membrane.

Several proteins encoded in the corrinoid biosynthesis cluster are not assigned to any function in corrinoid biosynthesis. The gene products of SMUL_1548 and SMUL_1567 are 10 and 8 kDa large proteins, containing eight cysteines each. Similar proteins (approximately 50% amino acid sequence identity) are found in a range of bacteria, primarily Fusobacteria, Firmicutes and Delta-, Epsilon- and Gammaproteobacteria. The gene product of SMUL_1548 was found in Py/PCE-SF and Fo/PCE-SF, while that of SMUL_1567 was not detected in any sample. The gene product of SMUL_1565, an MsbA-like protein, was only identified in Py/Fu-grown cells of *S. multivorans*.

The TetR-like transcriptional regulator downstream of the corrinoid biosynthesis cluster (SMUL_1569 and 1572) was not identified in any of the samples, which is in line with the observation that the corresponding gene is disrupted by a transposase (SMUL_1570 and 1571). Downstream of the corrinoid biosynthesis cluster and the *tetR* pseudogene, several genes are located encoding putative proteins for which participation in corrinoid biosynthesis is unproven. One of these proteins (SMUL_1573) was detected in PCE-grown cells only, while the other one (SMUL_1575, a putative FMN-binding protein) is found at >50-fold higher levels in PCE-grown cells than with fumarate or nitrate, regardless of the electron donor. Since close relatives of both proteins are found in *Ilyobacter polytropus*, which has a similar corrinoid biosynthesis cluster[6,17], these proteins might have a role in corrinoid biosynthesis in *S. multivorans*. The two other proteins (SMUL_1574, a putative FeS cluster and FMN containing protein, and SMUL_1576, a putative membrane protein) were not detected. The proteins involved in the biosynthesis of uroporphyrinogen III, the precursor for biosynthesis of corrinoids (SMUL_0533, SMUL_1083, SMUL_1902 and 1906), are not differentially regulated with PCE as electron acceptor. Interestingly, HemD (SMUL_0775) (uroporphyrinogen-III synthase) was quantified only with nitrate as electron acceptor.

PCE-induced proteins encoded outside the OHR region. Only few proteins encoded outside the OHR region were more abundant in cells grown with PCE compared to fumarate or nitrate. These proteins are encoded at different locations throughout the whole genome, but perform related biological functions. Several putative chaperones are among the most abundant proteins in PCE-grown cells (Table S4) but not in cells grown with other electron acceptors. Heat shock protein Hsp20 (SMUL_0547) was quantified at approx 20 to 60 times higher levels in all PCE-samples (Table S3), making it the second most abundant protein in Py/PCE-SF (Table S4). Chaperone HtpG (SMUL_ 2014) was detected in higher levels in PCE-grown cells as well (between 3 and 7-fold). This is in contrast to HtpG and Hsp20 in dechlorinating *Desulfitobacterium* spp[18,19], where instead chaperones GroEL/ES are present in a higher amount in organohalide-grown cells. The latter chaperones were quantified in similar amounts in all samples of *S. multivorans*. Several other stress-related proteins are also more abundant in PCE-grown cells. The 11th most abundant proteins in Fo/PCE-SF is a superoxide dismutase (SMUL_0529), present at elevated concentrations in all PCE-samples (3.1–7.4-fold). A glutathione peroxidase (SMUL_368), rarely encoded in epsilonproteobacterial genomes and closely related to the corresponding *Bacillus*-like enzyme, was only quantified in PCE-grown cells (Py/PCE-ME and Fo/PCE-SF). A number of flagellar proteins showed higher levels after PCE cultivation. Flagellin (SMUL_3194; 5.6 to 72-fold, $p < 0.05$) and the flagellar hook-associated protein FlgL (SMUL_1598; up to >50-fold, $p < 0.01$) were present at significantly altered levels (Table S3). Furthermore, FliA, the flagellar sigma factor (SMUL_0381) was found in higher levels (up to 7.3-fold) in Fo/PCE cells and was not quantified in Py/Fu or Py/Ni-grown cells.

Oxidative catabolism. Electron donors used for growth of *S. multivorans* in this study were formate and pyruvate. Formate is oxidized via a membrane-bound, molybdopterin-containing formate dehydrogenase (Fdh) not encoded in the OHR region[6,20]. The Fdh2 subunits FdhA2 (SMUL_2873) and FdhB2 (SMUL_2872) were quantified in high amounts in both, pyruvate (8.0 to 8.9 for FdhA2 in the ME) and formate-grown cells (9.1 to 9.3, Table S1). The membrane-integral cytochrome *b* subunit FdhI2 (SMUL_2871) was identified only in Fo/PCE-ME. The formate dehydrogenase-specific chaperon FdhX1 (SMUL_2875) and the formate dehydrogenase accessory protein FdhD (SMUL_2870) could be quantified in samples obtained from all conditions. Whereas FdhD is present in medium to high amounts (7.8 to 8.9) and the protein is more abundant in formate-grown cells, FdhX1 is present in low to medium amounts (6.3 to 8.0) and found to be present in higher levels in pyruvate-grown cells (Table S1). Furthermore, two additional formate dehydrogenases (Fdh1, SMUL_970 to 972 and Fdh3, SMUL_2899 to 2901) were quantified in Fo/Fu-grown cells, where a significant amount (8.4 and 8.1, respectively, for the catalytic subunits) was detected. Additionally, gene products of Fdh1 were identified in Py/PCE and Py/Ni-grown cells. A cytoplasmic formate dehydrogenase encoded by SMUL_0079 was quantified as a protein with low abundance (6.7 to 7.4) under all growth conditions applied (Table S1).

Besides formate, hydrogen can be used as electron donor by *S. multivorans*. The genome of *S. multivorans* harbors four gene clusters encoding NiFe hydrogenases, two of which can be classified as hydrogen-uptake hydrogenases[6]. Even though hydrogen was not chosen as a growth substrate in this study, a membrane-bound hydrogen-uptake hydrogenase (SMUL_1423 to 1425, MBH) was detected in high amounts (large subunit values ranging from 9.2 to 9.9 in the membrane extract) in all samples. Moreover, all accessory proteins required for the maturation of the Ni-Fe active site (namely HypBCDE) were found with the exception of HypA. The three other hydrogenases encoded in the genome of *S. multivorans* were either detected in only a part of the samples or not identified at all. A putatively hydrogen-evolving hydrogenase of *S. multivorans* (encoded by SMUL_2383 to 2392), similar to hydrogenase 3 (Hyc) and 4 (Hyf) as part of the formate hydrogen lyase of *E. coli*[21], was detected and quantified preferentially in cells grown with pyruvate as electron donor (Table S1). The second uptake hydrogenase was not detected at all, while only one subunit (out of four) of a second putative hydrogen-evolving hydrogenase was quantified in Fo/PCE cells exclusively.

For the oxidation of pyruvate as electron donor, two different enzymes were detected in this study, of which the pyruvate:ferredoxin/flavodoxin oxidoreductase (PFOR) was more abundant under all conditions. The PFOR was detected at increased concentrations (1.2 to 7.5-fold) in all fractions from cells grown with pyruvate compared to formate. The electron acceptor for pyruvate oxidation mediated by PFOR of *S. multivorans* might be either ferredoxin or flavodoxin[6]. Here, only one ferredoxin (encoded by SMUL_303) was more abundant in pyruvate-grown cells compared to formate (up to 6-times), while no flavodoxin was quantified in any of the samples (Table S1). The second pyruvate-oxidizing enzyme, pyruvate dehydrogenase (quinone), PoxB (SMUL_1703), was quantified in all samples of cells grown with pyruvate as electron donor. With formate as electron donor, it was only quantified in Fo/PCE-ME and Fo/Ni-SF (Table S1). Remarkably, *poxB* is exclusively found in *S. multivorans* among all Epsilonproteobacteria sequenced up to date (Genbank nr database as of June 2015) and is closely related (58% amino acid sequence identity) to the enzyme in *E. coli*[22].

S. multivorans encodes two different respiratory complex I forms on its genome. A typical ("standard") complex I (Nuo, encoded by SMUL_0508 to 0520) as found in most bacteria (e.g. *E. coli*) and another one specific for Epsilonproteobacteria (encoded by SMUL_0195 to 0208). The main difference between both complexes is the function of two cytoplasmic subunits (NuoEF or NuoEF-like proteins), which interact with NAD^+ in the former and with either ferredoxin or flavodoxin in the latter[23]. While up to eight subunits of the epsilonproteobacterial-type could be detected and maximally seven of them

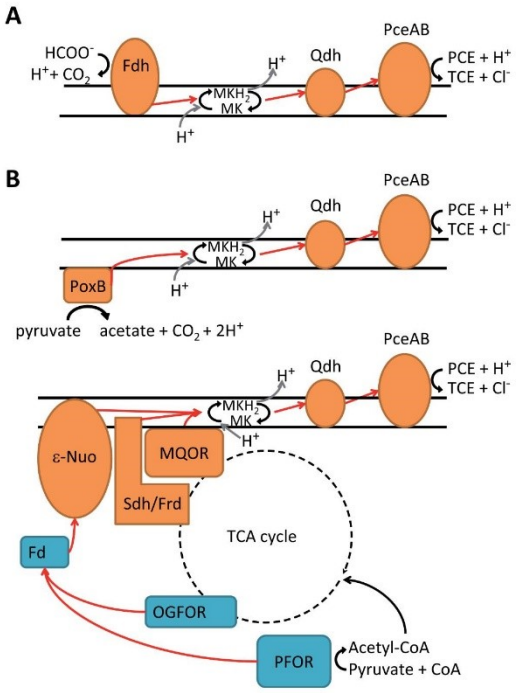

Figure 4. Overview of PCE-dependent catabolism in *S. multivorans*. (**A**) Formate-dependent PCE respiratory chain. (**B**) Pyruvate-dependent PCE respiratory chain, top: directly across the membrane employing quinone-reducing pyruvate dehydrogenase, bottom: cytoplasmic route of pyruvate oxidation via TCA cycle intermediates and ferredoxin. Abbreviations: Fdh, formate dehydrogenase; PoxB, pyruvate (quinone) dehydrogenase; Qdh, putative quinol dehydrogenase; PceAB, PCE reductive dehalogenase; PFOR, pyruvate:ferredoxin oxidoreductase; MQOR, malate:quinone oxidoreductase; Sdh/Frd, succinate dehydrogenase/fumarate reductase; OGFOR, 2-oxoglutarate:ferredoxin oxidoreductase; Fd, ferredoxin; ε-nuo, epsilonproteobacterial complex I; red arrows depict flow of electrons.

quantified, only one to four subunits of the "standard" complex I were identified, whereas in most conditions none of them were quantified (Table S1).

All enzymes of the ε-proteobacterial TCA cycle present in *Campylobacter jejuni*[24] were detected in medium to major amounts (approximately 8 to 10) under all conditions applied (Table S1). Malate is an important intermediate of the TCA cycle and numerous enzymes that can produce or oxidize malate are encoded in the genome of *S. multivorans*. They include two malate dehydrogenases (SMUL_0065 and 1443), a malate synthase (SMUL_0148), a malic enzyme (reversibly decarboxylating malate to pyruvate, SMUL_3158) and a malate-quinone oxidoreductase (SMUL_0667). Of these four proteins, the malate-quinone oxidoreductase is detected in major amounts in the membrane extract in all samples (8.7 to 9.2) and the malic enzyme in both, membrane and soluble extract (9.1 to 9.8). Of the two malate dehydrogenases (NAD^+-reducing), only SMUL_1443 was quantified in low to medium amounts (7.5 to 7.9). The malate synthase was quantified (7.2 to 7.8) in all pyruvate-grown cells, and in Fo/PCE-SF.

Nitrate respiration. The nitrate reductase operon is encoded by SMUL_0934 to 0940 (NapAGHBLD) and the ammonifying nitrite reductase operon by SMUL_0889 to 0892 (NrfHAIJ). Of the latter, only NrfA and NrfJ were identified or quantified. Both proteins were detected in most of the samples, regardless of the electron acceptor, although NrfJ was not identified in Fo/Ni-grown cells. In the latter cells, the catalytic subunit NrfA was detected in much higher amounts (six to >50 times) than in cells grown with PCE or fumarate as electron acceptor, whereas the difference in pyruvate-grown cells was negligible. The nitrate reductase catalytic subunit NapA (SMUL_0934) and cytochrome subunit NapB (SMUL_0937) were present in a higher amount in all nitrate-grown cells (3 to >50-times compared to PCE- or fumarate-grown cells). The periplasmic subunit NapG of the quinol dehydrogenase was quantified in Py/Ni-SF but not detected in any of the pyruvate-grown cells without nitrate. In Fo/Ni-grown cells, NapG

was more abundant (7 to 9-fold ratio) and NapH could be quantified in the membrane extract, opposed to the membrane-integral subunit of the PCE-induced quinol dehydrogenase (see above). One protein which is drastically more abundant in all nitrate-grown cells is a hydroxylamine reductase (SMUL_0602). While not identified in any of the cells grown without nitrate, it was among the most abundant proteins in both, Fo/Ni and Py/Ni-grown cells, where the amount was two to seven times higher in the soluble fraction than in the membrane extract.

Discussion

The differential proteomic analysis of *S. multivorans* cells grown under different substrate combinations allowed for the identification of components that might play a role in organohalide respiration of Epsilonproteobacteria including norpseudo-B_{12} biosynthesis, and it gives a comprehensive view on the basic catabolism of this free-living Epsilonproteobacterium.

The identification of 53% of the gene products annotated for *S. multivorans* provides a solid basis for comparative analysis of the different growth conditions. A large proportion of undetected proteins in this approach might not be synthesized as they may be required exclusively under different physiological conditions or during growth on other substrates.

Organohalide respiration and PCE stress response. The most remarkable differences in the analyzed proteomes of cells grown with and without PCE could be assigned to the OHR core region, which contains genes linked to organohalide respiration. Genes up- and downstream of this core are not induced with PCE, demonstrating that they do not play a role in PCE metabolism. The long-term down-regulation of PceA[6,12] is supported by the presented results.

Corrinoid biosynthesis proteins detected in this study were predominantly found in PCE-grown cells (Fig. 3), as opposed to *Desulfitobacterium hafniense* or *D. dehalogenans*, where an organohalide-dependent increased amount of these proteins has not been detected[19], except for CobT of *D. hafniense* TCE1 grown with PCE[18]. In *S. multivorans*, the norpseudo-B_{12} cofactor seems to be exclusively required for PceA synthesis under the tested conditions. The unique structural feature of the norpseudo-B_{12} produced by *S. multivorans* is the absence of methyl group 176 in the linker moiety of the nucleotide loop[9]. The *S. multivorans* enzyme involved in the nucleotide loop assembly pathway and most probably responsible for this difference is a distant homologue of CobD (SMUL_1544). Indeed, this enzyme was found in the proteome only in cells grown with PCE, further strengthening its role in production of norpseudo-B_{12}. Several proteins encoded in or downstream of the corrinoid biosynthesis gene cluster lack an assigned function. The cysteine-rich proteins SMUL_1548 and SMUL_1567 might be redox-active proteins through metal-binding or disulfide bridge formation and may aid processes in corrinoid biosynthesis. The PCE-dependent high abundance of SMUL_1573 and SMUL_1575, two presumably flavin-containing proteins of unknown function encoded by genes downstream of the corrinoid biosynthesis cluster is remarkable. It is feasible that flavin-containing enzymes help to sustain low redox states of the central cobalt during corrinoid biosynthesis[25]. PceB was found exclusively in membrane extracts of PCE grown cells, supporting the assumption that PceB is the membrane anchor of the PCE reductive dehalogenase[8] and that it is required for PCE respiration in *S. multivorans*. A second reductive dehalogenase, RdhA is encoded in the OHR region (SMUL_1536) and was identified only in formate/PCE-grown cells. The results clearly show that *rdhAB* is not induced with PCE as electron acceptor in general. Hence, the physiological function of this second reductive dehalogenase remains enigmatic.

In the OHR region, two genes (SMUL_1541 and SMUL_1542) encode a putative membrane-bound, periplasmic quinol dehydrogenase (Qdh)[6], of which SMUL_1541 was detected exclusively in PCE-grown cells. This points towards a participation of this Qdh in the PCE respiratory chain, transferring electrons from the menaquinone pool to PceA (Fig. 4), analogous to the role of NapGH in nitrate respiration[6,11,26]. Despite containing no membrane helices, SMUL_1541 was present exclusively in the membrane extract. This is likely due to a close interaction with the membrane-integral subunit of the Qdh (SMUL_1542), which was not detected in any sample, despite the previous detection of the corresponding mRNA in PCE-grown cells[6]. SMUL_1542, predicted to contain four membrane helices, was apparently not extracted from the membrane in this study. Candidates for the regulation of PCE respiration are the two-component regulatory systems downstream of each *rdh* cluster. Quantification of the response regulator encoded by SMUL_1539, downstream of the *rdhAB* cluster, indicates a participation of this protein in up-regulation of genes included in the OHR "core" region. This regulator was also identified and quantified in cells cultivated without PCE, which is in accordance with the putative function of SMUL_1539 in PCE-sensing, an ability that has to be maintained especially in the absence of PCE.

An alkylhydroperoxidase-like protein (SMUL_1530) was detected exclusively in PCE-grown cells. The protein is related (around 60% amino acid sequence identity) to a group of uncharacterized AhpD alkylhydroperoxidase-family proteins found in several other free-living, often marine Epsilonproteobacteria, Gammaproteobacteria, and Aquificales. The similarity to the characterized *Mycobacterium tuberculosis* alkylhydroperoxidase AhpD is low (~10%), but the catalytic residues [CxxCxxxH] are conserved[27]. As AhpD is a part of oxidative stress response in the latter bacterium, SMUL_1530 could function to protect proteins required in PCE metabolism from oxidative stress. In the proteome of the aerobic cDCE-degrading bacterium *Polaromonas* sp. JS666, an AhpC-like peroxidase was found in a higher amount in cells grown with cDCE[28]. As reductive (anaerobic) dechlorination is

highly unlikely to be carried out by this organism, a role of peroxidases in the general stress response to chlorinated ethenes seems feasible. A protein similar to IscU and NifU[29,30] (SMUL_1533) might have a function in the correct insertion of the FeS clusters in PceA[31]. Both proteins, the products of SMUL_1530 and 1533, are not found in other organohalide-respiring bacteria, therefore, their occurrence in *S. multivorans* may indicate a special role in PceA maturation in this organism.

Outside the OHR region, several proteins presumably involved in stress response were quantified in a larger amount in PCE-grown cells. This is in accordance with the fact that polychlorinated ethenes are toxic at elevated concentrations due to their high hydrophobicity. A non-characterized protein belonging to the Hsp20 heat shock family proteins (SMUL_0547), containing the so-called alpha-crystallin domain, was detected in higher amounts in PCE-grown cells. This protein family has been suggested to act ATP-independently as a multimeric chaperone, aiding in refolding proteins especially under stress[32,33]. A similar role might be fulfilled by HtpG, which was shown to have protein refolding activity in *E. coli*[34]. The higher abundance of a superoxide dismutase and glutathione peroxidase in PCE-grown cells is difficult to explain, as these are radical scavenging enzymes often involved in detoxifying reactive oxygen species[35,36]. However, it is assumed that superoxide dismutase may be also part of the general stress response mechanism as described for *B. subtilis*[37]. In *D. hafniense* TCE1, a catalase was found in higher amounts in PCE-grown cells[18], which might have a similar role in this organism. In PCE-grown cells of *S. multivorans,* these enzymes might be expressed either to detoxify radical intermediates formed in the process of reductive dechlorination[38,39] or as a component of the global stress response. The higher abundance of flagellar proteins, especially flagellin, might also be a part of the general stress response in *S. multivorans* or, alternatively, due to damage of the flagellum by chlorinated ethenes.

Oxidative catabolism. When formate serves as electron donor in *S. multivorans*, it is oxidized in the periplasm by a formate dehydrogenase, which donates electrons via a membrane-integral cytochrome *b* to the menaquinone pool. Therefore, the combination of formate as electron donor and PCE or nitrate as electron acceptor leads to a typical membrane-located respiratory chain (Fig. 4A). Of the three membrane-bound formate dehydrogenases encoded in the genome of *S. multivorans*[6], most probably Fdh2 (SMUL_2871 to 2873) is the main enzyme complex involved in respiratory formate oxidation[20]. The other two formate dehydrogenases seem to fulfill a backup formate oxidation system, since they were detected only in cells grown with formate and fumarate. The fact that Fdh2 is present in pyruvate-grown cells might be due to a constitutive expression. This might also apply to the membrane-bound hydrogenase (MBH), since the latter was detected under all growth conditions in high amounts. A similar situation was observed in several *Desulfitobacterium* spp.[19,40]. The constitutive expression of Fdh2 and MBH points towards the importance of both formate and hydrogen as electron donors in *S. multivorans* which might be among the preferred available electron donors in natural habitats of *Sulfurospirillum* spp. such as sludge or sediments. The second hydrogenase which was quantified in high amounts is the presumably hydrogen-evolving Hyf hydrogenase. It might function as an electron sink for excess reduced ferredoxin produced by PFOR in case of electron acceptor limitation. Additionally, the presence of a hydrogen-producing enzyme may explain the high amount of MBH which is possibly responsible for hydrogen recycling.

The route of electrons generated by pyruvate oxidation to the terminal electron acceptor is not clear in *S. multivorans*. Pyruvate could be oxidized via two enzymes. One of them is the typical epsilonproteobacterial PFOR, which most likely reduces a ferredoxin encoded by SMUL_0303. This is opposed to the situation in the Epsilonproteobacterium *Helicobacter pylori*, where presumably flavodoxin is interacting with the PFOR[41]. An ortholog of this flavodoxin (SMUL_2785) was not detected in this study. In the Firmicutes *D. hafniense* Y51, PFOR was also suggested to transfer electrons from pyruvate to ferredoxin[40]. In *S. multivorans,* the reduced ferredoxin could be oxidized by the epsilonproteobacterial complex I, which then transfers electrons to the quinone pool (Fig. 4B). The second enzyme used for pyruvate oxidation by *S. multivorans* is pyruvate (quinol) dehydrogenase, a cytoplasmic lipoenzyme which interacts directly with the quinone pool[22,42]. This enzyme seems to be mainly synthesized in pyruvate-grown cells, but as high amounts of the PFOR are present in cells grown with pyruvate as electron donor, both enzymes could be responsible for pyruvate oxidation (Fig. 4B).

Nitrate respiration. The response of *S. multivorans* to nitrate as electron acceptor is of interest, since nitrate respiration parallels PCE respiration in the way that both systems contain a periplasmic terminal reductase and most probably involve a quinol dehydrogenase. Although single steps of respiratory nitrate ammonification and the intermediates formed have not yet been determined for *S. multivorans*, we have been able to identify enzymes and reactions likely involved in this pathway by comparative proteome analysis of cells grown with nitrate, PCE, or fumarate. The higher abundance of the nitrate reductase NapAB and the quinol dehydrogenase NapGH in nitrate-grown cells points towards a nitrate-dependent regulation of nitrate respiratory chain proteins. However, the high amount of NapAB in formate-grown cells without nitrate indicates a global regulation of nitrate respiratory enzymes dependent on the carbon and energy source. Highly significant is the large amount of a hydroxylamine reductase (hybrid cluster protein) in all nitrate-grown cells. Hydroxylamine is presumably not released as reaction intermediate during nitrate ammonification in contrast to denitrification[43] nor is hydroxylamine reductase part of the stress response to hydroxylamine in the closely related *Wolinella succinogenes*[44]. Recently, an enzyme

complex was reported to be responsible for hydroxylamine production in the Epsilonproteobacterium *Nautilia profundicula*[45], but the central enzyme of this complex (the periplasmic hydroxylamine oxidoreductase) is not encoded in the genome of *S. multivorans*. However, the hydroxylamine reductase of *S. multivorans* might function as scavenger of other intermediates of nitrate ammonification such as nitrite or NO as reported for other bacteria[46,47]. Therefore, it can be assumed that the increased level of the hydroxylamine reductase is part of a global response to the presence of nitrate.

Conclusion

This proteome analysis sheds light on components linked to the organohalide-respiratory chain, proteins involved in maturation thereof and the global response to PCE and other energy substrates in the Epsilonproteobacterium *S. multivorans*. A NapGH-like quinol dehydrogenase was identified as the most probable link between the quinone pool and the reductive dehalogenase PceA. In this respect, PCE respiration resembles Nap-mediated nitrate respiration. A NifU/IscU-like protein not observed in other organohalide-respiring bacteria before might be specific for FeS cluster assembly of PceA. Detection of the norpseudo-B_{12} biosynthesis machinery exclusively in PCE-grown cells confirms the crucial role of the corrinoid cofactor for PCE respiration. A peroxidase-like protein might protect PceA against oxidative stress. The global response to PCE involves an increase in the production of chaperones, which differ from those found in dechlorinating *Desulfitobacterium* spp. This study also provides first insights into the electron transfer from pyruvate to PCE including the potential involvement of a quinone-reducing pyruvate dehydrogenase and of a pyruvate:ferredoxin/flavodoxin oxidoreductase presumably reducing the ferredoxin SMUL_0303. An epsilonproteobacterial complex I mediates the electron transfer from ferredoxin to the quinone pool.

All in all, these findings contribute to a deeper insight into organohalide respiration and might help to better understand the general bacterial response to these widely distributed but environmentally harmful substances. Furthermore, this proteome analysis might aid the research on the hitherto underexplored ecophysiology of free-living Epsilonproteobacteria.

Methods

Cultivation of *S. multivorans*. *S. multivorans* (DSMZ 12446) was cultivated under anaerobic conditions at 28 °C in a defined mineral medium[4] without vitamin B_{12} (cyanocobalamin). Pyruvate (40 mM) or formate (40 mM) were used as electron donor and fumarate (40 mM), nitrate (10 mM with formate, 40 mM with pyruvate as electron donor) or PCE as electron acceptor. PCE was added to the medium (10 mM nominal concentration) from a hexadecane stock solution (0.5 M). When the cells were grown with formate, acetate (5 mM) was added as carbon source and the medium contained 0.05% yeast extract to support growth. Titanium(III)-citrate (5.6 μM) was added when *S. multivorans* was grown with formate and nitrate (10 mM). Cultivation of *S. multivorans* was performed using the following substrate combinations: pyruvate/fumarate (Py/Fu), pyruvate/PCE (Py/PCE), pyruvate/nitrate (Py/Ni), formate/fumarate (Fo/Fu), formate/PCE (Fo/PCE) and formate/nitrate (Fo/Ni). Pre-cultures were grown in rubber-stoppered 200 mL glass serum bottles and the main cultures in rubber-stoppered 2 L glass bottles. The ratio of aqueous to gas phase was always 1:1. In order to generate *S. multivorans* cells with down-regulated *pceA* gene expression[12], the organism was cultivated for 60 subcultivation steps (10% inoculum, each) on pyruvate (40 mM) and fumarate (40 mM) plus 0.2% yeast extract. The last culture was used as inoculum for all pre-cultures. The inoculum corresponded to approximately 18 μg/ml cell protein for Py/Fu-grown cells, 12 μg/ml for Fo/Fu cells, 6 μg/ml for Py/PCE and Py/Ni-grown cells and 3 μg/ml cell protein in the case of Fo/Ni and Fo/PCE cultures. The bacterial growth was monitored photometrically by measuring the optical density at 578 nm. All cultivations were performed in triplicates.

Cell harvesting and samples preparation. *S. multivorans* cells were harvested in the late exponential growth phase by centrifugation (12,000 × g, 10 min at 10 °C). The cell pellets were washed once in 50 mM Tris-HCl (pH 7.5) and resuspended (1:2) in the same buffer containing a tip of a spatula of DNase I (AppliChem, Darmstadt, Germany) and protease inhibitor (one tablet for 10 ml buffer; cOmplete Mini, EDTA-free; Roche, Mannheim, Germany). The cells were disrupted using a French Press (1000 psi). Cell debris was removed by centrifugation (6,000 × g, 10 min at 4 °C). The supernatant was ultracentrifuged (260,000 × g, 45 min at 4 °C) and the resulting supernatants were decanted (soluble protein fractions = SF). The pellets (membrane extract) were washed twice with 50 mM Tris-HCl (pH 7.5) including protease inhibitor and finally resuspended in solubilization buffer (1% (w/v) Digitonin (AppliChem, Darmstadt, Germany), 300 mM NaCl, 100 mM Tris-HCl, pH 7.5). The membrane protein extracts were obtained by stirring overnight at 16 °C. After ultracentrifugation (260,000 × g rpm, 45 min at 4 °C), the supernatants (membrane protein extracts = ME) were collected and analyzed (Fig. 1).

Immunoblot analysis. Soluble fractions (10 μg protein per lane) were subjected to denaturing SDS PAGE (12.5%) and afterwards blotted onto a polyvinylidene difluoride (PVDF) membrane (Roche, Mannheim, Germany) using a semi-dry transfer cell (Bio-Rad, Munich, Germany) according to the protocol described by John *et al.* (2009). The PceA antiserum (primary antibody) was diluted 500,000-fold.

The primary antibody was detected via a secondary antibody (diluted 1:20,000) coupled to alkaline phosphatase (Sigma-Aldrich, Munich, Germany).

Measurement of PceA activity. *S. multivorans* cells were harvested in the late exponential growth phase by centrifugation (12,000 × g, 10 min at 10 °C) under air. Cell pellets were washed with 50 mM Tris-HCl (pH 7.5). The cell pellets were transferred into an anoxic glove box and resuspended (1:2) in anoxic buffer (50 mM Tris-HCl, pH 7.5). An equal volume of glass beads (0.25–0.5 mm diameter, Carl Roth GmbH, Karlsruhe, Germany) was added and the cells were disrupted using a bead mill (5 min at 25 Hz; MixerMill MM400, Retsch GmbH, Haan, Germany). The crude extracts were separated from the glass beads by centrifugation (14,000 × g, 2 min) under anoxic conditions. The measurements of PceA activity were performed as described before using a photometric assay with reduced methyl viologen as artificial electron donor[7].

SDS-PAGE and proteolytic digestion. The protein concentration was determined after protein extraction using the Bio-Rad Bradford reagent (Bio-Rad, Munich, Germany) and bovine serum albumin as protein standard. Protein samples of solubilized membrane extract and soluble fractions were subjected to SDS-PAGE. Per lane, 50 µg of protein lysates was applied. Afterwards, each sample lane was cut into four slices and prepared for proteolytic cleavage using trypsin (Promega, Madison, WI, USA)[48]. Peptide lysates were extracted and desalted using C18 ZipTips (Merck Millipore, Darmstadt, Germany).

Mass spectrometry and proteome data analysis. Separation of tryptic peptides was performed using an Ultimate 3000 nanoRSLC system (Thermo Scientific, Germering, Germany) coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific, San Jose, CA, USA). A sample volume of 1 µL was loaded onto a trapping column with 300 µm inner diameter, packed with 5 µm C18 particles (µ-precolumn, Thermo Scientific) and separated via a 15 cm analytical column (Acclaim PepMap RSLC, 2 µm C18 particles, Thermo Scientific). The column oven temperature was set to 35 °C. During the liquid chromatography (LC) run, a constant flow of 300 nL/min (solvent A: 0.1% formic acid) was applied with a linear gradient of 4% to 55% solvent B (80% acetonitrile, 0.08% formic acid) in 90 min. Mass spectrometer (MS) full scans were measured in the Orbitrap mass analyzer within the mass range of 400–1,700 m/z at 60,000 resolution using an automatic gain control target of 4×10^5 and maximum fill time of 50 ms. The MS analyzed in data-dependent acquisition mode; the highest intense ions with positive charge states between 2 and 7 were selected for MS/MS. An MS/MS isolation window for ions in the quadrupole was set to 1.6 m/z. MS/MS scan were acquired within 3 s of cycle time (Top Speed) using the higher energy dissociation mode at normalized collision induced energy of 35%, activation time of 120 ms, and minimum of ion signal threshold for MS/MS of 5×10^4 counts. The exclusion time to reject masses from repetitive MS/MS fragmentation was set to 30 s.

LC-MS/MS data were analyzed using Proteome Discoverer (v1.4.1.14, Thermo Scientific). MS/MS spectra were searched against the data of the *S. multivorans* database containing 3,191 non-redundant protein-coding sequence entries (downloaded February 17th 2014 from NCBI Genbank accession number CP007201) using the SEQUEST HT and MS Amanda algorithms with the following settings: trypsin as cleavage enzyme, oxidation of methionine as dynamic and carbamidomethylation of cysteine as static modification, up to two missed cleavages, MS mass tolerance set to 10 ppm and MS/MS mass tolerance to 0.05 Da, respectively. False discovery rate for peptides was <0.01 (Supplement information Table S1). Quantification of proteins was performed using the average of top 3 peptide area[49]. Protein quantification was considered successful for proteins quantified in >50% of biological replicates, otherwise they were classified as identified proteins. After log10 transformation, the protein values were normalized and bioinformatic analysis was applied by principal component analysis and T-test statistics (R). Significance threshold $p < 0.05$ in a two-tailed test were considered as significantly altered.

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Acknowledgement

This work was supported by the German Research Foundation (DFG), as part of the research group FOR 1530. C.L.S. was also supported by Helmholtz Impulse and Networking Fund through the Helmholtz Interdisciplinary Graduate School for Environmental Research (HIGRADE). The authors are grateful for being able to use the analytical facilities of the Centre for Chemical Microscopy (ProVIS) at the Helmholtz Centre for Environmental Research, which is supported by European Regional Development Funds (EFRE - Europe funds Saxony) and the Helmholtz Association. Benjamin Scheer is thankfully acknowledged for technical assistance and maintenance of the Orbitrap Fusion Mass Spectrometer.

Author Contributions

G.D., T.S., J.S. and M.v.B. initiated the study, T.G., G.D. and N.J. supervised the study. T.G., C.L.S. and J.G. drafted the manuscript and analyzed the data. C.L.S. conducted the mass-spectrometrical analysis and preparation of the protein samples, J.G. performed wet lab work with the cells as well as the enzymatic analysis. T.G., C.L.S., J.G., N.J. and G.D. discussed and interpreted the data. All authors revised the draft manuscript and read and approved the final manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Goris, T. *et al.* Proteomics of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates. *Sci. Rep.* **5**, 13794; doi: 10.1038/srep13794 (2015).

Supplemental material to:

Proteomics of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates

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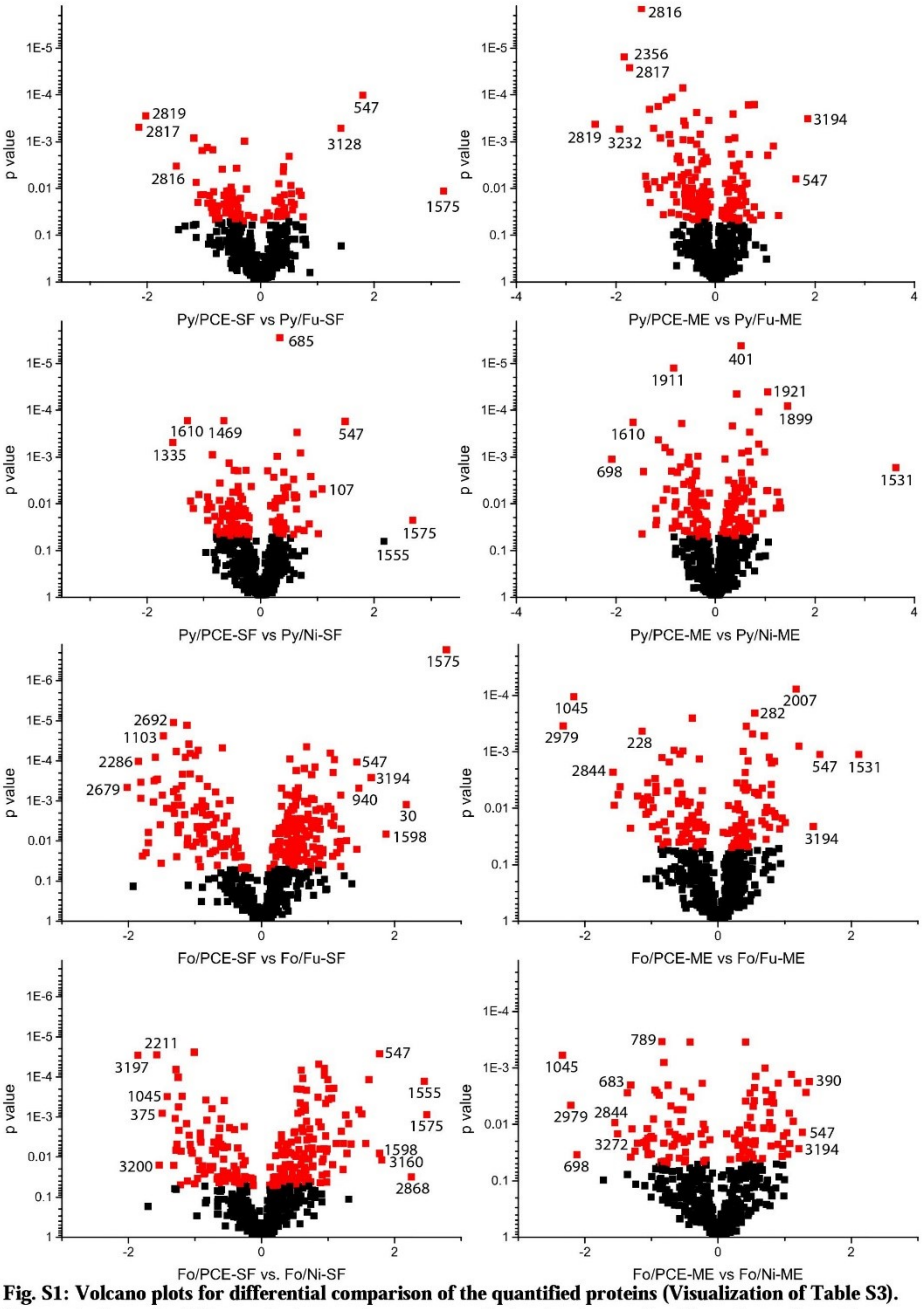

Fig. S1: Volcano plots for differential comparison of the quantified proteins (Visualization of Table S3). The x-axis shows the difference in the protein amount as calculated and normalized from the top 3 peptide area (as described in materials and methods). Values are shown as log10. A value of more than 0.477 is taken as significant (equals an approximately 3-fold amount). The y-axis shows statistical significance as a result from the T-test. A p-value of less than 0.05 is taken as significant (marked in red). Numbers of the SMUL_locus tags are given for the proteins with the most significant changes (see Table S3).

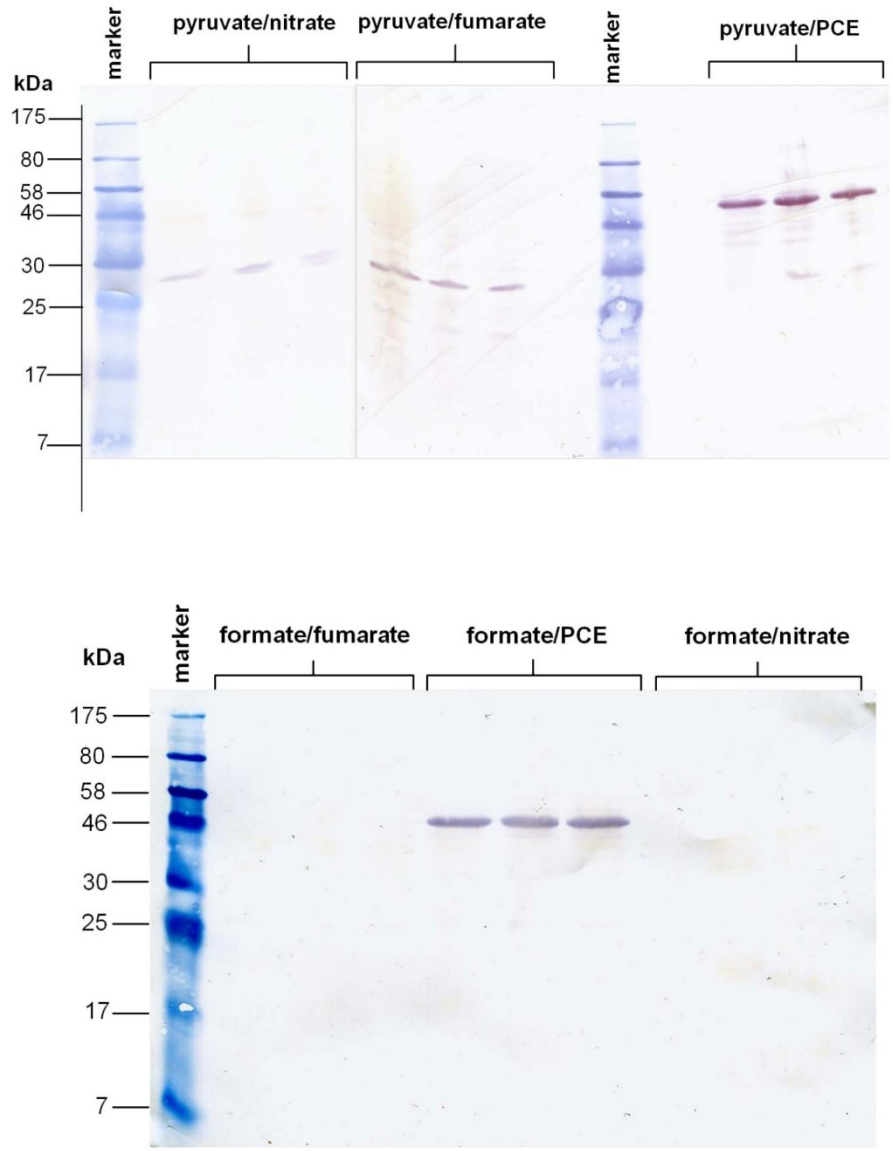

Fig. S2: Western-Blots: Anti-PceA (1:500.000). 10 µg protein from all biological replicates which were used in the proteome analysis was applied per lane. PceA is visible at approximately 50kDa. The band visible in samples from pyruvate-grown cells without PCE at around 27kDa is an unknown protein product, possibly degraded PceA. Blots were applied to tonal correction.

Table S5: PceA activity. The PceA activities were measured photometrically as described in the materials and methods section.

Sample	$Volume_{sample}$ [µl]	volume activity [nkat/ ml]	Specific activity [nkat/ ml]	GC-measurement (increase of cDCE)
Pyruvate/Fumarate	100	1,328	0,320	-
Pyruvate/PCE	5	213,752	20,183	++
Pyruvate/Nitrate	100	10,2067	1,180	-
Formate/Fumarate	100	3,9683	0,4663	+
Formate/PCE	5	75,9717	20,812	++
Formate/Nitrate	100	-	-	-

Supplemental Tables

Due to the size of the data sets, the supplemental tables were not printed. Here is a brief description of the contents of the single tables. The corresponding links to the tables are given.

Table S1

This table collects the basic proteomic datasets from all samples in 13 sheets. "Data norm" lists the normalized and logarithmized (log10) data of all single samples in one sheet. Proteins which were not identified in any of the samples are not listed.
The sheets for each of the samples lists the values of the area under the curve for the single triplicates plus the number of identified peptides and unique peptides for each protein

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4585668/bin/srep13794-s2.xls

Table S2

This table lists the proteins which were either identified or quantified in at least on sample extracted from PCE-grown cells and was neither identified nor quantified in any of the samples extracted from cells grown with fumarate or nitrate as electron acceptor.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4585668/bin/srep13794-s3.xls

Table S3

This table includes the differential comparison of samples extracted from PCE-grown cells versus fumarate or nitrat. This data was used as the basis for the volcano plots (Fig. S2). Data were logarithmized and normalized before included in this table.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4585668/bin/srep13794-s4.xls

Table S4

This table contains the proteins quantified as the 20 most abundant in each sample.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4585668/bin/srep13794-s5.xls

3.3 Manuskript III

Data in Brief 8 (2016) 637–642

Contents lists available at ScienceDirect

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Data Article

Proteomic data set of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates

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ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form
9 June 2016
Accepted 15 June 2016
Available online 21 June 2016

Keywords:
Anaerobic respiration
Epsilonproteobacteria
Nitrate respiration
Reductive dechlorination
Reductive dehalogenase

ABSTRACT

Sulfurospirillum multivorans is a free-living, physiologically versatile Epsilonproteobacterium able to couple the reductive dehalogenation of chlorinated and brominated ethenes to growth (organohalide respiration). We present proteomic data of *S. multivorans* grown with different electron donors (formate or pyruvate) and electron acceptors (fumarate, nitrate, or tetrachloroethene [PCE]). To obtain information on the cellular localization of proteins, membrane extracts and soluble fractions were separated before data collection from both fractions. The proteome analysis of *S. multivorans* was performed by mass spectrometry (nanoLC-MS/MS). Raw data have been deposited at ProteomeXchange, "*ProteomeXchange provides globally coordinated*

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http://dx.doi.org/10.1016/j.dib.2016.06.022

proteomics data submission and dissemination" [1], via the PRIDE partner repository with the dataset identifier PRIDE: PXD004011. The data might support further research in organohalide respiration and in the general metabolism of free-living Epsilonproteobacteria. The dataset is associated with a previously published study "Proteomics of the organohalide-respiring Epsilonproteobacterium *S. multivorans* adapted to tetrachloroethene and other energy substrates" [2].

Specifications Table

Subject area	Microbiology
More specific subject area	Proteomics
Type of data	Table
How data was acquired	Ultimate 3000 RSLC (Thermo Scientific Dionex) coupled with a TriVersa NanoMate (Advion Biosciences, Norwich, UK) to an Orbitrap Fusion mass spectrometer (Thermo Scientific).
Data format	Raw
Experimental factors	Prior LC-MS/MS measurement
Experimental features	1) Cultivation of bacteria 2) Protein extraction 3) LC-MS/MS analysis
Data source location	Leipzig, Saxony, Germany
Data accessibility	Data are within this article. The mass spectrometry proteomics data have been deposited at ProteomeXchange via the PRIDE partner repository with the dataset identifier PRIDE: PXD004011.

Value of the data

- Data set can be used for the characterization of organohalide respiration in Epsilonproteobacteria and to elucidate the electron transport chains between the electron donors formate or pyruvate and the electron acceptors fumarate, nitrate, or tetrachloroethene as well as the general stress response to chlorinated ethenes.
- The wide range of anaerobic substrates used in this study helps to get insights into the regulation of anaerobic respiration with emphasis on organohalide respiration in Epsilonproteobacteria.
- LC-MS/MS data provides a comprehensive proteome of *S. multivorans* including the information about the abundance of proteins in different nutritional conditions and can help to unravel anaerobic metabolism of free-living Epsilonproteobacteria.
- Separated analysis of the soluble and membrane fraction can deliver valuable information about cellular localization of closely related proteins in other Epsilonproteobacteria.

1. Data

In this dataset, we present the proteome of the organohalide-respiring, free-living Epsilonproteobacterium *Sulfurospirillum multivorans* grown with different electron donors (pyruvate or formate)

and electron acceptors (fumarate, nitrate, or tetrachloroethene [PCE]). The data presented here are uploaded to PRIDE and contains i) LC-MS/MS data (*.raw) and ii) database search files (*.msf) of 36 LC-MS/MS measurements (three replicates of each, soluble fraction and membrane extract) of *S. multivorans* cultivated under 6 different anaerobic conditions (pyruvate with PCE, nitrate, or fumarate; formate with PCE, nitrate, or fumarate). The provided data are summarized in Table 1.

2. Experimental design, materials and methods

Here, we investigated the soluble/membrane proteome of *S. multivorans* in response to different energy substrate with different substrate combinations. Pyruvate/fumarate (Py/Fu) was used as the "standard" growth condition, the results of which were compared to the following electron donor/acceptor combinations: pyruvate with nitrate (Py/Ni) or PCE (Py/PCE); formate with fumarate (Fo/Fu), PCE (Fo/PCE) or nitrate (Fo/Ni). We explored the downregulation of the organohalide respiratory machinery in *S. multivorans* when the cells were cultivated for a long time (about 100 generations) in the absence of chlorinated ethenes [3]. For the generation of these “organohalide-respiration-silent” cells, pyruvate/fumarate was used as substrate combination for 60 transfers. The whole set was prepared for LC-MS/MS analysis and the acquired data are provided.

3. Growth of Sulfurospirillum multivorans

The defined mineral medium used for growth of *S. multivorans* (German Collection of Microorganisms [DSMZ] number 12446) was prepared as previously described [4]. Oxygen was removed with 30 cycles gas evacuation and flushing with molecular nitrogen. The following modification to the original medium was used: The medium was prepared without cyanocobalamin (vitamin B_{12}). The cultivation of *S. multivorans* was performed at 28 °C under continuous shaking in rubber-stoppered 2 L glass flasks (Schott, Germany). Precultures for inoculation of the main cultures were grown in rubber stoppered 200 mL serum bottles; transfers were always performed with 10% culture volume. All precultures were inoculated with cultures grown with pyruvate and fumarate for 60 transfer steps to generate cells lacking proteins involved in organohalide respiration [3]. Before inoculation of the main culture, the precultures were transferred three consecutive times into the desired medium with a 10% inoculum in order to avoid transfer of pyruvate or fumarate from the initial preculture. The ratio between medium and gas phase (v/v) during all growth experiments was 1 to 1. The electron donors pyruvate and formate and the electron acceptors fumarate and nitrate were used at a concentration of 40 mM, each, except in the combination of formate and nitrate, where nitrate was supplied at a concentration of 10 mM. PCE was dissolved in hexadecane (0.5 M stock solution). Concentration of PCE in the medium was 10 mM. Acetate (5 mM) was added as carbon source when formate was used as electron donor and those media were supplemented with 0.05% yeast extract. Titanium(III)-citrate (5.6 μM) was added when *S. multivorans* was grown with formate and nitrate. Overall, six electron donor/acceptor conditions were used: the standard condition pyruvate/fumarate (Py/Fu) and five conditions for comparison: pyruvate/nitrate (Py/Ni), pyruvate/PCE (Py/PCE), formate/fumarate (Fo/Fu), formate/nitrate (Fo/Ni) and formate/PCE (Fo/PCE).

4. Cell harvesting and sample preparation

Cells of each growth condition were grown in 3×1 L medium to the late exponential growth phase and then harvested via centrifugation at 12,000 g for 10 min at 10 °C. The cell pellets were washed and resuspended in a ratio of 1:2 in 50 mM Tris HCl buffer (pH 7.5). One tip of a spatula DNase I (AppliChem, Darmstadt, Germany) and one tablet protease inhibitor (cOmplete Mini, EDTA-free; Roche, Mannheim, Germany) per 10 mL buffer were added. Cell disruption was performed with a French Press at 1000 psi (6.89 MPa) followed by removal of cell debris by centrifugation at 6000*g* for 10 min at 4 °C. The resulting supernatant was ultracentrifuged (260,000x*g* for 45 min at 4 °C) and the

Table 1
Sulfurospirillum multivorans proteome data that are provided at PRIDE. For each condition three biological samples were processed.

Cultivation condition			**Sample name for LC-MS/MS and database search file** (numbers indicates the replicates)
Electron donor	**Electron acceptor**	**Extract/ Fraction**	
Pyruvate (Py)	tetrachloroethene (PCE)	Soluble	*_Smult_PyPCELE1
			*_Smult_PyPCELE2
			*_Smult_PyPCELE3
		Membrane	*_Smult_PyPCEME1
			*_Smult_PyPCEME2
			*_Smult_PyPCEME3
Pyruvate (Py)	nitrate (Ni)	Soluble	*_Smult_PyNOLE1
			*_Smult_PyNOLE2
			*_Smult_PyNOLE3
		Membrane	*_Smult_PyNOME1
			*_Smult_PyNOME2
			*_Smult_PyNOME3
Pyruvate (Py)	fumarate (Fu)	Soluble	*_Smult_PyFuLE1
			*_Smult_PyFuLE2
			*_Smult_PyFuLE3
		Membrane	*_Smult_PyFuME1
			*_Smult_PyFuME2
			*_Smult_PyFuME3
Formate (Fo)	tetrachloroethene (PCE)	Soluble	*_Smult_FoPCELE1
			*_Smult_FoPCELE2
			*_Smult_FoPCELE3
		Membrane	*_Smult_FoPCEME1
			*_Smult_FoPCEME2
			*_Smult_FoPCEME3
Formate (Fo)	nitrate (Ni)	Soluble	*_Smult_FoNOLE1
			*_Smult_FoNOLE2
			*_Smult_FoNOLE3
		Membrane	*_Smult_FoNOME1
			*_Smult_FoNOME2
			*_Smult_FoNOME3
Formate (Fo)	fumarate (Fu)	Soluble	*_Smult_FoFuLE1
			*_Smult_FoFuLE2
			*_Smult_FoFuLE3
		Membrane	*_Smult_FoFuME1
			*_Smult_FoFuME2
			*_Smult_FoFuME3

resulting soluble protein fraction (SF) in the supernatant was carefully decanted. The residual pellet (membrane fraction) was washed twice with protease inhibitor amended washing buffer (see above) and afterwards resuspended in solubilization buffer (300 mM NaCl, 100 mM Tris–HCl, pH 7.5) containing 1% (w/v) digitonin (AppliChem, Darmstadt, Germany) as detergent. This mixture was stirred overnight at 16 °C and subsequently ultracentrifuged (260,000xg, for 45 min at 4 °C). The resulting supernatants are designated as membrane protein extracts (ME).

5. Proteomics by short SDS-PAGE

We determined the protein amount using the Bio-Rad Bradford assay (Bio-Rad, Munich, Germany). 50 μg protein lysates were applied to run shortly (1.5 cm) on a 12% SDS-PAGE (1.0 mm thickness, running condition: 10 mA for 10 min followed by 20 mA for 20 min). Each sample lane was cut into four pieces of about 5 mm each, which were prepared for proteolytic cleavage by reduction and alkylation separately. Protein digestion was carried out with sequencing-grade trypsin (Promega, Madison, WI, USA). The resulting peptides were desalted and extracted with C_{18} ZipTips (Merck Millipore, Darmstadt, Germany). The four peptide lysates from one sample lane were unified before LC-MS/MS analysis.

6. Mass spectrometry and data analysis

Mass spectrometric analysis was performed by separation of tryptic peptides using an Ultimate 3000 nanoRSLC (Thermo Scientific, Germany) system coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific, San Jose, CA, USA). We used two mobile phases: mobile phase A was 0.1% formic acid, mobile phase B contained 80% acetonitrile and 0.08% formic acid. Peptides were loaded for 5 min on the precolumn (μ-precolumn, cartridge column, 3 μm particle size, 75 μm inner diameter, 2 cm, particles C_{18}, Thermo Scientific) at 4% mobile phase B and eluted from the analytical column (PepMap Acclaim C_{18} LC Column, 15 cm, 3 μm particle size, Thermo Scientific) over a 90 min gradient of mobile phase B (4-55% B). The MS setting were described in detail [2].

The software Proteome Discoverer (v1.4.1.14, Thermo Scientific) was used for protein identification and the acquired MS/MS spectra were searched with the Sequest HT and MS Amanda algorithms against the *S. multivorans* genome database with 3191 non-redundant CDS (as of February, 17th 2014 available from the NCBI Genbank database, accession number CP007201, [5]). Settings of Sequest HT and MS Amanda were as follows: trypsin digestion, dynamic oxidation of methionine and carbamidomethylation of cysteine as fixed. Up to two missed cleavages were allowed, MS mass tolerance was set to 10 ppm and the MS/MS mass tolerance to 0.05 Da. The quantitative information of the proteins was calculated by the average of the top three peptides per protein with the precursor ion area detector implemented in the Proteome Discoverer. The database search result files (*.msf) are also uploaded to PRIDE and can be accessed.

Acknowledgements

This work was supported by the German Research Foundation (DFG), as part of the research unit FOR 1530. CLS was also supported by Helmholtz Impulse and Networking Fund through the Helmholtz Interdisciplinary Graduate School for Environmental Research (HIGRADE). The authors are grateful for the use of the analytical facilities of the Centre for Chemical Microscopy (ProVIS) at the Helmholtz Centre for Environmental Research, which is supported by European Regional Development Funds (EFRE - Europe funds Saxony) and the Helmholtz Association. Benjamin Scheer is thankfully acknowledged for technical assistance and maintenance of the Orbitrap Fusion mass spectrometer.

Transparency document. Supplementary material

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dib.2016.06.022.

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3.4 Manuskript IV

Investigations on the tetrachloroethene respiratory chain of *Sulfurospirillum multivorans*

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Abstract

Tetrachloroethene (PCE) respiration was studied in the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans*, with respect to the involvement of menaquinone in the electron transport and the requirement of a reverse electron flow to drive this respiration. The dechlorination of PCE in cell suspensions of *S. multivorans* was inhibited by the menaquinone antagonist 2-n-heptyl-4-hydroxychinolin N-oxide (HQNO), showing that menaquinone is involved in the electron transport within the PCE respiratory chain. The requirement of a reverse electron flow was demonstrated by using the protonophores Carbonyl cyanide-p-(trifluoromethoxy)-phenylhydrazone (FCCP) and Carbonyl cyanide-m-chlorophenyl hydrazone (CCCP). Both protonophores caused the inhibition of organohalide respiration in cell suspensions of *S. multivorans*. For comparison the nitrate and fumarate respiration of *S. multivorans* were also examined. Menachinone is involved in the electron transport of both respiratory chains, whereas neither nitrate nor fumarate respiration is dependent on a reverse electron flow. In *S. multivorans*, two different menaquinones could be identified under different cultivation conditions tested: menaquinone-6 and methylmenaquinone-6, with menaquinone-6 being identified as the major quinone

Introduction

Hazardous halogenated organic compounds are man-made products of industrial origin as well as products of geogenic and biogenic sources and therefore abundant in nature since billions of years (Gribble, 2010; Atashgahi *et al.*, 2016). As a consequence, microorganisms have adapted to exploit these compounds as substrates in the course of the evolution (Smidt & de Vos, 2004; Hug, 2016). Halogenated hydrocarbons can be dehalogenated by a special type of anaerobic respiration, which couples reductive dehalogenation to energy conservation via electron transport phosphorylation (organohalide respiration - OHR) (Leys *et al.*, 2013). To date, OHR is described for different phyla of the bacterial domain, such as Proteobacteria, Firmicutes and Chloroflexi (Atashgahi *et al.*, 2016). Organohalide-respiring bacteria (OHRB) are classified according to two metabolic strategies: obligate or non-obligate organohalide respirers.

The gram-negative ε-Proteobacterium *Sulfurospirillum multivorans*, a non-obligate OHRB able to reductively dehalogenate tetrachloroethene (PCE) and trichloroethene (TCE) to cis-1,2-dichloroethene (cDCE) using different electron donors (e.g. hydrogen, formate, pyruvate, lactate) (Goris & Diekert, 2016). The key enzyme of PCE respiration is the reductive dehalogenase (PceA),

which harbors a Tat (twin-arginine translocation) signal peptide, two [4Fe-4S] clusters as well as a corrinoid cofactor at the active site. PceA is attached to the periplasmic face of the cytoplasmic membrane most probably via the putative membrane-anchor protein PceB. Though PceA is well characterized (Neumann *et al.*, 1996; Bommer *et al.*, 2014), the electron transport chain, responsible for the electron transfer between the electron donor and the reductive dehalogenase, is not completely elucidated until now. The involvement of menaquinones (Goris *et al.*, 2014) as well as a reverse electron flow (Miller *et al.*, 1996) in the PCE respiratory chain is suggested.
Quinones are a key component of aerobic as well as various anaerobic respiratory chains, where they act as both electron and hydrogen carriers. As a non-obligate OHRB, *S. multivorans* is also able to use fumarate, nitrate and oxygen as electron acceptors in addition to PCE (Goris & Diekert, 2016). The involvement of quinones in the fumarate and nitrate respiratory chain has already been demonstrated for other Epsilonproteobacteria, e.g. for the gram-negative Epsilonproteobacterium *Wolinella succinogenes* (Kröger *et al.*, 2002). A convenient way to study the involvement of quinones in electron transport within respiratory chains are inhibition studies with quinone analogues as it was shown for the fumarate reductase of *Escherichia coli* (Maklashina & Cecchini, 1999). The inhibition of PCE respiration in *S. multivorans* by the use of different protonophores - Carbonyl cyanide-p-(trifluoromethoxy)-phenylhydrazone (FCCP) and tetrachlorosalicylanilide (TCS) – was previously shown (Miller *et al.*, 1997). The inhibition is supposed to be based on the dissipation of the reverse electron flow across the membrane, which is assumed to be an essential component of the PCE respiratory chain. The necessity of this reverted electron flow probably results from the energetic relationships of the electron transport chain of PCE-respiration as are currently known.

To elucidate the architecture and mechanism of the PCE respiratory chain of *S. multivorans*, several approaches were used: First, 2-n-heptyl-4-hydroxychinolin N-oxide (HQNO), an effective and potent ubiquinone and menaquinone antagonist was used to investigate the involvement of quinones in PCE respiration and, as a comparison, nitrate and fumarate respiration. HQNO acts on many respiratory cytochrome *b*-containing redox enzymes (Smirnova *et al.*, 1995) as well as those lacking heme such as the menaquinol-fumarate reductase of *Escherichia coli* (Cecchini *et al.*, 1995; Rothery & Weiner, 1998) by binding to the specific quinone binding site. Second, protonophores such as FCCP were used to study the involvement of a reverse electron flow and third, menaquinones of *S. multivorans* should be identified. Other Epsilonproteobacteria and another non-obligate OHRB, *Desulfitobacterium hafniense* Y51 (Furukawa *et al.*, 2004), were used for comparison.

Material and Methods

Cultivation of *Sulfurospirillum* spp. and Desulfitobacterium hafniense Y51. *S. multivorans* (DSMZ 12446), *S. halorespirans*, *S. deleyianum* and were cultivated under anaerobic conditions at 28 °C in a defined mineral medium (Scholz-Muramatsu *et al.*, 1995) without vitamin B_{12} (cyanocobalamin). Pyruvate (40 mM), lactate (40 mM), formate (40 mM), hydrogen (100% in the gas phase at 150 kPa) or oxygen (5% in the gas phase) were used as electron donors and fumarate (40 mM), nitrate (40 mM) or PCE as electron acceptors. PCE was added to the medium (10 mM nominal concentration) from a hexadecane stock solution (0.5 M). When the cells were grown with formate or hydrogen, acetate (5 mM) was added as carbon source. *D. hafniense* Y51 was cultivated in the same medium, but with yeast extract (0.2%).

Cultivation of *Wolinella succinogenes*. *Wolinella succinogenes* cells were grown by nitrate respiration as described in Kern *et al.* (2007) with formate (80 mM) as electron donor and nitrate (50 mM) as electron acceptor at 37°C.

The pre-cultures as well as the main cultures of all used organisms were grown in rubber-stoppered 200 mL glass serum bottles under anoxic conditions. The ratio of aqueous to gas phase was 1:1. The bacterial growth was monitored photometrically by measuring the optical density at 578 nm. Cells were harvested anoxically in the late exponential growth phase by centrifugation (12,000 x g, 10 min at 10°C).

Effect of inhibitors on different anaerobic respiratory chains. The effect of inhibitors on different anaerobic respirations was studied in cell suspensions. For the preparation of cell suspensions, the cells were grown and harvested as described above. The bacteria were suspended in MOPS-KOH buffer (100 mM, pH 7.5). Experiments were performed at 28°C respectively 37°C in rubber stoppered serum bottles (25 ml), N_2 was the gas phase. Where indicated, 2-n-heptyl-4-hydroxychinolin N-oxide (HQNO; 320 nmol/mg protein or 640 nmol/mg protein), Carbonyl cyanide-p-(trifluoromethoxy)-phenylhydrazone (FCCP; 15 nmol/mg protein or 30 nmol/mg protein), Carbonyl cyanide-m-chlorophenyl hydrazone (CCCP; 15 nmol/mg protein or 30 nmol/mg protein) or N-cetyl-trimethylammonium bromide (CTAB; 0.01%; w/w) were added. Cells were incubated with inhibitors for one hour before adding the substrates required for the respective experiment.

Analytical Methods. Protein concentrations were determined in accordance to the method described by Bradford (1976) using the Bio-Rad reagent (Bio-Rad Laboratories, Munich, Germany). Bovine serum albumin was used as standard.

Nitrite was measured by a diazotization reaction described by Lunge (1904). The colorimetric reaction was started by the addition of 50 µl of 1% (w/v) sulphanilic acid and 50 µl of 0.3% (w/v) 1-naphthylamine to 50 µl of the diluted sample. After 5 min incubation at room temperature, absorption was measured at a wavelength of 525 nm on a VERSAmax tunable microplate reader (Molecular Devices, Biberach an der Riss, Germany). For nitrate determination, the method of Bosch Serrat (1998) was followed. Diluted samples were mixed in a 1:1 ratio with 200 µl of a chloride solution consisting of 0.28 M NaCl dissolved in 9.35% (v/v) H_3PO_4. Subsequently, 1 ml of 0.55% (w/v) resorcinol dissolved in 62.5% (v/v) H_2SO_4 was added to each sample. The mixtures were incubated at 95 °C for 8 min. They were allowed to cool down for 20 min and absorbance was measured at 505 nm. In this assay both nitrite and nitrate were detected, therefore nitrate concentration was calculated by subtracting the nitrite concentration measured in the first assay from the value obtained.

For pyruvate determination, the method of Friedemann & Haugen (1942) was followed. Diluted samples (300 µl) were mixed with 100 µl 2,4-Dinitrophenylhydrazine (1 mg/ml). After 5 min incubation at room temperature, 500 µl NaOH (2.5 N) were added to the mixtures. Absorption was measured at a wavelength of 520 nm on a VERSAmax tunable microplate reader (Molecular Devices, Biberach an der Riss, Germany).

Fumarate conversion was measured by HPLC analysis. Liquid samples were filtered with 0.2 µm-syringe filters (MiniSart RC4, Satorius, Göttingen, Germany) and acidified with concentrated H_2SO_4 (2.5 µl/ml sample volume). Organic acids were separated at 50°C on an AMINEX HPX-87H column (7.8 x 300 nm; BioRad, Munich, Germany) with a cation H guard pre-column using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.7 ml/min. The injection volume was 20 ml per sample. Fumarate was identified by its absorption at 210 nm as well as at 240 nm. Retention time (11.89 min) was compared to known fumarate standard and concentrations were calculated using a calibration curve.

PCE, TCE and cDCE amounts were measured with gas phase GC-FID analysis of cell suspension samples using a Clarus 500 gas chromatograph as described in Mac Nelly *et al.* (2014). Concentrations were calculated using calibration curves.

Quinone analysis. *S. multivorans* cells used for quinone analysis were grown and harvested as described above. Pyruvate (40 mM) served as electron donor and fumarate (40 mM), PCE and oxygen (5%) as electron acceptors. Total lipid analysis was performed as previously reported (White & Ringelberg, 1998). Total lipids were extracted from lyophilized cells (5 g; 7 hours at 7 x 10-3 Pa) with the one-phase chloroform-methanol-buffer system of Bligh & Dyer (1954) as

modified by White et al. (1996). Lyophilized medium samples served as control blanks to account for exogenous source of lipids.

In a first step the total lipid was extracted by suspending the cells in a mixture (ratio 1:2:0.8) of chloroform, methanol and 50 mM phosphate buffer (pH 7.4; the buffer was rinsed with chloroform) and following sonication (10 min in the ultrasonic bath) of the samples. The samples were incubated overnight at 28°C, shaking at 100 rpm. After overnight Bligh and Dyer extraction, equal volumes of chloroform and nanopure water were added to the extract, resulting in a two-phase system. The lower organic phase (containing lipids) was collected and concentrated to dryness under a gentle stream of nitrogen. Total lipid residues were resolved in 200 µl chloroform and fractionated by silicic acid column chromatography into neutral lipids, glycolipids and polar lipids by sequential elution with 5 ml chloroform, 5 ml aceton and 10 ml methanol, respectively, into separate glass vials. All three lipid fractions were dried with nitrogen with nitrogen and stored at -20°C. For respiratory quinone analysis the neutral lipid fraction of Bligh and Dyer extract after fractionation on silicic acid columns was examined by HPLC/APCI/MS/MS using a quadrupole-linear iontrap mass spectrometer in the MRM mode (Multiple Reaction Monitoring Mode). The samples were solved in 100 µl methanol. 10 µl of the samples were analyzed.

Results

Quinones as an essential component of the PCE respiratory chain of *S. multivorans*

Identification of quinones present in anaerobic respiratory chains of *S. multivorans*

Analysis of quinones was carried out for different cultivation conditions in order to determine possible changes in the quinone composition in response to the growth substrates: pyruvate served as electron donor while PCE, fumarate or oxygen served as electron acceptors. In *S. multivorans*, two different menaquinones could be identified under all cultivation conditions tested: menaquinone-6 and methylmenaquinone-6 (Figure 1), with menaquinone-6 being identified as the major quinone. Both quinones have 6 isoprene residues in the side chain. The position of the methyl group of methylmenaquinone-6 in the aromatic ring was not determined. Menaquinone-6 and methylmenaquinone-6 were also found in *W. succinogenes* (Collins & Fernandez, 1984).

Involvement of menaquinone in different anaerobic respirations

To show the involvement of menaquinone in PCE, nitrate and fumarate respiration of *S. multivorans,* the effect of HQNO (2-n-heptyl-4-hydroxychinolin N-oxide), an inhibitor of menaquinone-dependent redox-reactions, was investigated in cell suspension experiments. In all experiments two HQNO concentrations were tested – 320 nmol/mg protein and 640 nmol/mg protein, and a negative control without HQNO.

The effect of HQNO on the PCE respiration was tested with cell suspensions of *S. multivorans* (see Materials and Methods). Pyruvate or formate were used as electron donors. For comparison, the experiments were also conducted with *Desulfitobacterium hafniense* Y51, another well-known PCE-dechlorinating bacterium (Furukawa *et al.*, 2004). Because *D. hafniense* Y51 was not able to use formate as electron donor in the cell suspension experiments, only pyruvate was used as substrate. In all experiments PCE served as electron acceptor.
In *S. multivorans* HQNO completely inhibited PCE reduction with formate (supplemental Figure 2A) or pyruvate as electron donors (Table 1). Formation of cDCE and TCE could not be detected in the reaction medium. In contrast, the PCE respiration of *D. hafniense* Y51 was not affected by 320 nmol/mg protein HQNO (supplemental Figure S1) as well as by 640 nmol/mg protein (supplemental Table S2). For *S. multivorans* HQNO concentrations of 50 nmol/mg protein and 100 nmol/mg protein led to no, respectively 10% inhibition of PCE respiration. Taken together,

menaquinone seems to be an essential component of the PCE respiratory chain of *S. multivorans* but not of the PCE respiratory chain of *D. hafninense* Y51. This is in contrast to another study, where the involvement of menaquinone in the ortho-chlorophenol respiration of *Desulfitobacterium dehalogenans* was shown by analyzing the redox state of quinones extracted from the organism (Kruse *et al.*, 2015). Therefore, HQNO inhibition was tested for fumarate respiration of *D. hafniense* Y51, which was already demonstrated for the fumarate respiration of *Wolinella succinogenes* (Kröger *et al.*, 2002).

In *S. multivorans* the fumarate respiration with formate as electron donor was inhibited 54% with 320 nmol/mg protein and by 89% with 640 nmol/mg protein HQNO (supplemental Figure S2 A, Table 1). When pyruvate served as electron donor no inhibition of fumarate-respiration could be observed (Table 1). The fumarate respiration of *D. hafniense* Y51 was not affected by HQNO (supplemental Figure S2 B; supplemental Table S1). Because of this results it can be assumed that HQNO is not a suitable inhibitor for investigating the involvement of menaquinones in fumarate respiratory chains when pyruvate serves as electron donor.

The effect of HQNO on the nitrate respiration was tested with cell suspensions of *S. multivorans* and *W. succinogenes* (see Materials and Methods). Formate served as electron donor and nitrate as electron acceptor. For *S. multivorans* the experiments were also performed with pyruvate as electron donor.
In *S. multivorans* (Figure 2B) and also in *W. succinogenes* (Figure 2C) HQNO almost completely inhibited nitrate reduction with formate and pyruvate as electron donors (Table 1).

A reverse electron flow as an essential component of the PCE respiratory chain of *S. multivorans*

The protonophore FCCP (Carbonyl cyanide-p-(trifluoromethoxy)-phenylhydrazone) was used to investigate the involvement of a reverse electron flow in different anaerobic respiratory chains of *S. multivorans*. FCCP is a hydrophobic proton carrier and as such abolishes the electrochemical membrane potential by carrying protons across the gradient without generating energy. In a previous study it was shown that FCCP has no negative effect on hydrogenase, reductive PCE dehalogenase and fumarate reductase activities of *S. multivorans*, which excludes an inhibitory effect of FCCP on these enzymes (Miller *et al.*, 1996).

The inhibition experiments were performed in cell suspensions. In all experiments two FCCP concentrations were tested – 15 nmol/mg protein and 30 nmol/mg protein – and a control without FCCP was carried along.

For both tested FCCP concentrations, complete inhibition of PCE respiration was observed in *S. multivorans* over a period of 210 min (Figure 3A). The inhibition was independent of the chosen electron donors - pyruvate, formate, lactate and hydrogen (Table 1). The experiment was repeated with *S. halorespirans* (Luijten *et al.*, 2003), a closely related OHRB to *S. multivorans*. Here, also a complete inhibition of PCE respiration was shown for the tested substrate combination formate/PCE (supplemental Table S1). In contrast, no inhibition of PCE-respiration by FCCP could be observed for *D. hafniense* Y51 (Figure S3, supplemental Table S1) with pyruvate as electron donor. Also, the increase in FCCP concentration to 90 nmol/mg protein did not lead to inhibition.

Nitrate and fumarate respiration *S. multivorans* (Figure 3B, Table 1) as well as nitrate respiration of *W. succinogenes* (Figure 3C) were not inhibited by the tested FCCP concentrations when formate was used as electron donor. In contrast the nitrate and fumarate respiration of *S. multivorans* were completely or partially inhibited by the tested FCCP concentrations when pyruvate served as electron donor (supplemental Figure S4; Table 1). A similar inhibitory effect was observed for *S. halorespirans* and the non-dechlorinating *S. deleyianum* (supplemental Table S1). To exclude the possibility of an inhibitory effect of FCCP on the enzymes pyruvate:ferredoxin oxidoreductase and pyruvate dehydrogenase which are involved in pyruvate oxidation in *S. multivorans*, the effect of the protonophore on pyruvate fermentation was tested. Only slight inhibition of FCCP (around 8% for both tested concentrations) on pyruvate fermentation was observed (supplemental Figure S5), which cannot explain the strong inhibition in fumarate and nitrate respiration.

The experiments were repeated with carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) which acts similar to FCCP. The results were the same with all electron donor substrate combinations in all organisms (data not shown). N-cetyl-trimethylammonium bromide (CTAB), a cationic surfactant let to the inhibition of all respiratory chains (data not shown), probably due to its function which causes leaky cell membranes.

Discussion

In this communication, the inhibition of different anaerobic respiratory chains in the OHRB *S. multivorans* and related bacteria by several functionally distinct inhibitors was studied. The results aid in elucidating architecture and function of PCE respiration in comparison to nitrate and fumarate respiration.

In *S. multivorans*, two different menaquinones – menaquinone-6 and methylmenaquinone-6- were identified. Both menaquinones found in *S. multivorans* were also detected in the non-dechlorinating strain EK7, which is a species within the genus *Sulfurospirillum* somewhat different from the type species *S. deleyianum* (Ballerstedt *et al.*, 2004), while in the strict organohalide-respiring bacteria *Dehalobacter restrictus* PER-K23 menaquinone-6 was detected besides menaquinone-7, menaquinone-8 and menaquinone-9 (Maillard & Holliger, 2016). For *W. succinogenes* it was shown that menaquinone-6 is a central component of the fumarate respiratory chain while methylmenaquinone-6 is a component of the polysulfide respiratory chain (Dietrich & Klimmek, 2002). The involvement of menaquinone in the electron transport from the electron donor to the electron acceptor within different respiratory chains of *S. multivorans* was shown with the menaquinone antagonist HQNO. PCE, nitrate and fumarate respiration could be inhibited by HQNO when formate or hydrogen served as electron donor. (Table 1). The inhibition of the investigated respirations was probably due to the binding of HQNO to the cytochrome *b* subunit of the formate dehydrogenase (FdhC) (Figure 4) respectively hydrogenase (HydC) as it has already by shown for the formate dehydrogenase and hydrogenase of *W. succinogenes* (Biel *et al.*, 2002; Kröger *et al.*, 2002). In contrast, the fumarate respiration was not inhibited by HQNO when pyruvate was used as electron donor (Table 1). This might be due to the fact, that the two cytoplasmic enzymes, responsible for the pyruvate oxidation in *S. multivorans* – the pyruvate:ferredoxin oxidoreductase (PFOR) and the pyruvate (quinol) dehydrogenase (PoxB) – as well as the epsilonproteobacterial complex I and the fumarate reductase are probably insensitive to HQNO. The epsilonproteobacterial complex I is involved in the electron transfer from PFOR to the menaquinone pool, while the PoxB interacts directly with the menaquinone pool (Goris *et al.*, 2015). The fumarate reductase of *W. succinogenes*, which is similar to the one of *S. multivorans* (Goris *et al.*, 2014), was already shown to be insensitive to HQNO (Lemma *et al.*, 1991; Kröger *et al.*, 2002). Nitrate and PCE respiration were inhibited by HQNO even when pyruvate was used as electron donor (Table 1). Hence, in this two respirations other components, affected by HQNO, must be involved in the electron transport within the respiratory chains. These components are discussed in explaining the tentative schemes of the different respiratory chains.

A tentative scheme of the topology of the PCE respiratory chain of *S. multivorans* grown with formate as electron donor and PCE as electron acceptor is depicted in Figure 4A. The formate dehydrogenase and hydrogenase as well as the reductive dehalogenase PceA are localized in the periplasm (Schmitz & Diekert, 2003; Miller *et al.*, 1997; John *et al.*, 2006), while POXB and PFOR are cytoplasmic proteins (Goris *et al.*, 2015). PceA is attached to the cytoplasmic membrane by the putative membrane anchor PceB. Until now there is no indication for a direct interaction of PceA with the menaquinone pool in the cytoplasmic membrane. Hence, the involvement of an additional component for the quinol oxidation and transfer of the electrons to the terminal reductase is expected. In *S. multivorans* two proteins SMUL_1541 and SMUL_1542, showing similarities to the putative quinol dehydrogenase NapGH of *W. succinogenes* (Goris *et al*., 2014; Goris *et al*., 2015), were identified as possible components responsible for the electron transport between the menaquinone pool and PceA. NapGH might be involved in the electron transport from the menaquinone pool to the terminal reductase in nitrate respiration, the periplasmic NapA (Kern & Simon, 2008). The NapG-like protein encoded by SMUL_1541 contains four [4Fe-4S] cluster binding motifs and an N-terminal Tat signal peptide. Therefore the protein is considered to be localized in the periplasm while the NapH homologue – SMUL_1542 – harboring two [4Fe-4S] cluster binding motifs is predicted to contain four membrane-spanning helices when predicted to topology prediction (Goris *et al.*, 2014). Another component which might be involved in PCE respiration is a small putative membrane protein (SMUL_1540) with three predicted transmembrane helices, exclusively found in PCE-grown cells (Goris *et al.*, 2014; Goris *et al.*, 2015). Due to the results, namely the inhibition of PCE respiration with HQNO when pyruvate served as electron donor (Table 1), we assumed that HQNO might bind to one or both membrane-integral proteins, SMUL_1542 and SMUL_1540, probably involved in organohalid respiration. A major thermodynamic problem still exists in the reduction of the [Cob^{II}] to [Cob^{I}] in the course of the catalytic reaction cycle. The [Cob^{II}]/[Cob^{I}] couple has a midpoint redox potential of -380 mV (pH 7.5, versus a standard hydrogen electrode) (Bommer *et al.*, 2014). Furthermore a redox potential of around -450 mV was determined for both Fe/S clusters of PceA (Siritanaratkul *et al.*, 2016). With menaquinone (E_{SHE} = -74 mV) respectively methylmenaquinone (E_{SHE} = -174 mV) being the electron donor for the reduction of the Fe/S clusters and therefore the reduction of the cobalamin, the input of metabolic energy is required for the [Cob^{II}] reduction. The results of this study as well as data obtained earlier (Miller *et al.*, 1996), namely the inhibition of PCE respiration by using protonophores (FCCP, CCCP), demonstrated that the electron required for this thermodynamically unfavorable reduction is driven via a reverse electron flow. At which point the reverse electron flow takes place could not be determined until now.

So we wanted to investigate the PCE respiration of *S. multivorans* the PCE respiratory chain of *D. hafniense* Y51 was examined for comparison. Pyruvate served as electron donor in all inhibition experiments because *D. hafniense* Y51 was not able to use formate as electron donor in cell suspensions, though the organism is described as formate consumer (Villemur *et al.*, 2006). The involvement of menaquinone in PCE respiration of *D. hafniense* Y51 could not be shown by using HQNO (supplemental Table S1). However, the involvement of menaquinone in PCE respiration of *D. hafniense* Y51 cannot be excluded because for *Desulfitobacterium dehalogenans*, the involvement of menaquinone in organohalid respiration was demonstrated (Kruse *et al.*, 2015). The redox state of quinones, extracted before and after incubation of *D. dehalogenans* cells with 3-chloro- 4-hydroxyphenyl acetate (Cl-OHPA), were analyzed. An increase in the oxidized form of the quinones after incubation with Cl-OHPA was seen as indication that the quinones are involved in the electron transport within the organohalid respiratory chain. This was a more indirect method of investigation than inhibition experiments, which allows to examine the involvement of quinones in respiratory chains in which HQNO has no binding sites. Fumarate respiration of *D. hafniense* Y51 remained also unaffected by HQNO (supplemental Table S1 and figure S2B), which might be due to the reason explained above for the fumarate respiration of *S. multivorans* with pyruvate as electron donor. Other organohalide respiring bacteria (OHRB) than *S. multivorans* for which the involvement of menaquinone in there organohalide respiratory chains was demonstrated by using HQNO are *Dehalobacter restricuts* (Schumacher & Holliger, 1996) and *Desulfomonile tiedjei* (Louie & Mohn, 1999). In *D. hafniense* Y51 no reverse electron flow is required to drive the organohalide respiration, as is also the case for all other examined OHRB until now except the organohalide respiring *Sulfurospirillum (S. multivorans* and *S. halorespirans*, see results). Therefore it can be assumed that organohalide respiration may be completely different in the organohalide respiring bacteria with respect to energy conservation and to the electron carriers involved.

Tentative schemes of the topology of the nitrate- and fumarate-respiratory chains of *S. multivorans* grown with formate as electron donor are depicted in Figure 4B and 4C. The nitrate respiratory chain shows topological similarities to the PCE respiratory chain with the terminal reductase (NapA) localized in the periplasm and a putative quinol dehydrogenase (NapGH) involved in the electron transport from the electron donor, via the menaquinone pool, to the electron acceptor. In contrast the terminal reductase of fumarate respiration the fumarate reductase is localized in the cytoplasm. The nitrate respiratory chain of *W. succinogenes* corresponds to the nitrate respiratory chain of *S. multivorans.*

The fumarate- and the nitrate-respiration of *S. multivorans* remained unaffected by the protonophore FCCP when formate served as electron donor (Table 1; results). The same was true for the nitrate-respiration of *W. succinogenes*. The results corresponded to our expectations. Until now, for none of the investigated fumarate respectively nitrate respiratory chains the requirement of a low-potential electron donor and therefore a reverse electron flow was reported. The fumarate-respiration of *W. succinogenes* is one of the most extensively studied anaerobic respirations known today and though the fumarate reductase of *S. multivorans* is similar to the one described for *W. succinogenes* (Goris *et al.*, 2014) we assume that fumarate respiration in *S. multivorans* might function in a similar way as it is predicted for *W. succinogenes*. The menaquinone, present in the membrane of *S. multivorans*, is reduced by formate while the protons consumed in menaquinone reduction are probably taken up from the cytoplasmic side of the cells (Figure 4C) as it is predicted for the fumarate respiration of *W. succinogenes* (Kröger *et al.*, 2002). The reduced menaquinone can then easily be reoxidized by the cytochrome *b* subunit of the fumarate reductase (E_{SHE} = -20 mV; Unden *et al.*, 1980; Kröger *et al.*, 2002). For *S. multivorans*, no experimental data exist on which side of the membrane the protons are released during the menachinol oxidation. The potential difference between formate and menaquinone drives the electrons across the membrane in fumarate respiration.

In the respiratory chain from formate to nitrate (Figure 4B), the proton motive force is assumed to be generated by a redox loop mechanism during menaquinone reduction by formate, catalyzed by the formate dehydrogenase as it was already shown for the nitrate respiration of *W. succinogenes* (Kern & Simon, 2009). The enzymes NapG and NapH probably form a membrane-bound complex that might catalyse menaquinol oxidation and subsequent electron transfer to the terminal reductase NapA. In contrast to fumarate-respiration nitrate-respiration is also affected by HQNO when pyruvate served as electron donor (Table 1). This might be because of the proposed binding of HQNO to the membrane-integral subunit – NapH – of the putative quinol dehydrogenase, involved in nitrate-respiration (see above).

Unexpected was the inhibition of the fumarate and nitrate respiration of *S. multivorans* by the protonophore FCCP when pyruvate served as electron donor (supplemental Figure S4, Table 1). A slight inhibition of pyruvate oxidation by FCCP could be observed (supplemental Figure S5), but not to the extent that it would explain the complete inhibition of nitrate respiration respectively the extensive inhibition of fumarate respiration. Due to the results it can be assumed that the dissipation of the membrane potential or the pH gradient (or both) caused by FCCP somehow interferes with the electron transport from the electron donor pyruvate to the electron acceptors fumarate respectively nitrate. How this is done could not be determined in this study.

Conclusions: *S. multivorans* harbors two different types of menaquinones – menaquinone-6 and methylmenaquinone-6 – which function as electron carriers in the different respiratory chains of *S. multivorans*. This was shown for the PCE, fumarate and nitrate respiration by using the menaquinone antagonist HQNO. Furthermore it was proven that the PCE respiration of *S. multivorans* requires a reverse electron flow to drive the electron transport within the organohalide respiratory chain while in contrast the fumarate and nitrate respiration of *S. multivorans* do not involve a reverse electron flow.

Funding: This work was supported by the German Research Foundation (DFG), as part of the research unit FOR 1530. The authors used the analytical facilities of the Centre for Chemical Microscopy (ProVIS) at the Helmholtz Centre for Environmental Research, which is supported by European Regional Development Funds (EFRE - Europe funds Saxony) and the Helmholtz Association.

Acknowledgement: Benjamin Scheer is thankfully acknowledged for technical assistance and maintenance of the Orbitrap Fusion mass spectrometer.

Main Figures and Tables

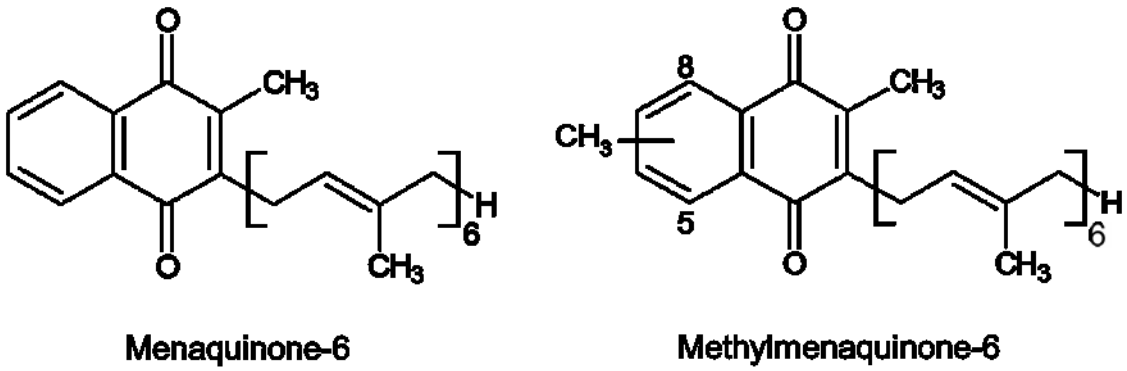

Figure 1: Structure of the identified quinones: menaquinone-6 and methylmenaquinone-6. Quinones were extracted from *S. multivorans* cells grown with pyruvate as electron donor and PCE, fumarate or oxygen as electron acceptor and were identified by HPLC/APCI/MS/MS using a quadrupole-linear iontrap mass spectrometer in the MRM mode.

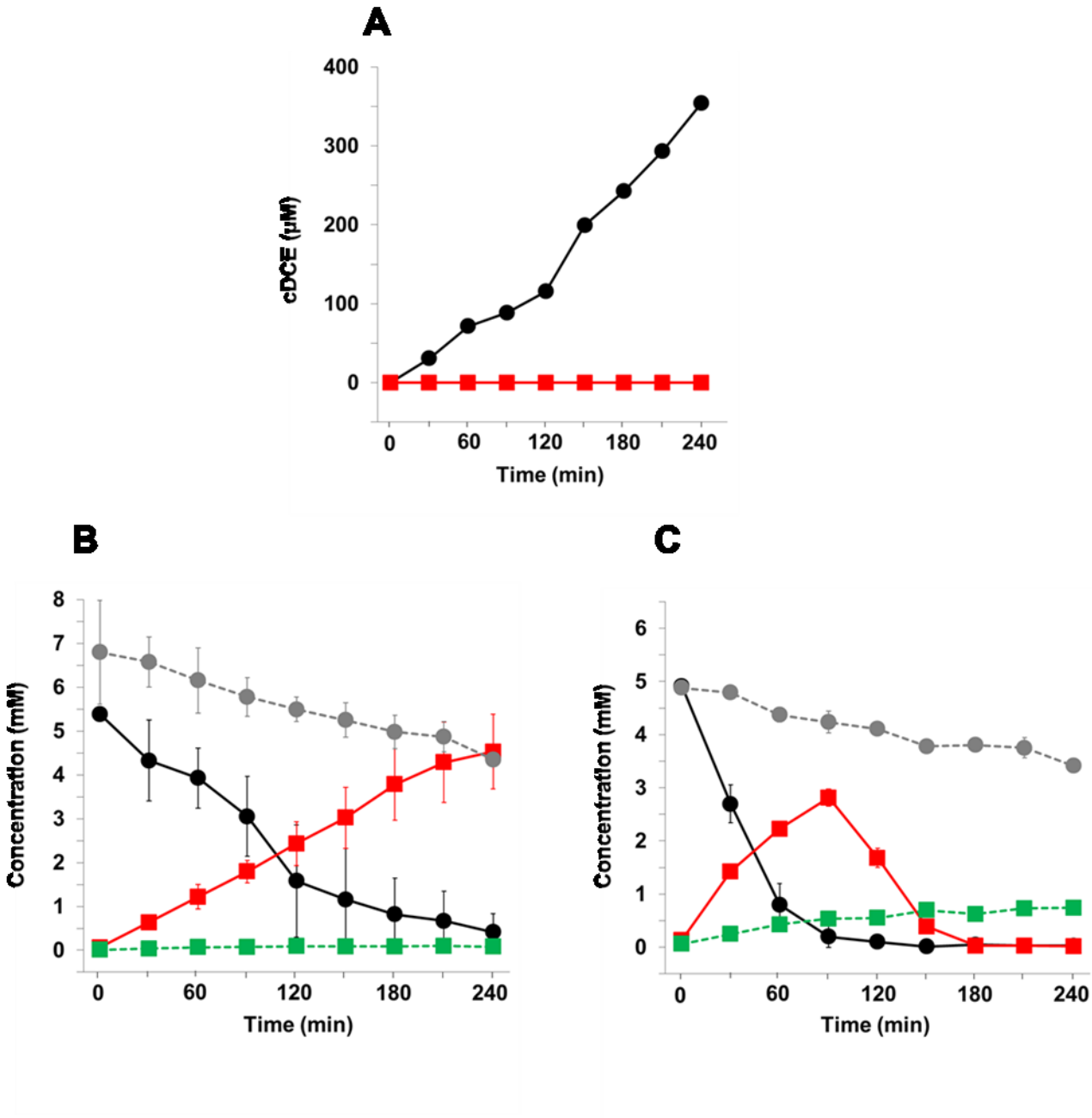

Figure 2: Effect of HQNO on PCE and nitrate respiration in cell suspensions of *S. multivorans* and *W. succinogenes*. A: PCE respiration of *S. multivorans*. Depicted is the formation of cDCE in the presence respectively absence of HQNO. Black circles: control without HQNO; red squares: + HQNO. **B / C: Nitrate respiration of *S. multivorans* (B) and *W. succinogenes* (C)**. Depicted is the reduction of nitrate (initially 5mM) and the formation of nitrite in the presence or absence of HQNO. Black circles: nitrate reduction in the absence of HQNO; red squares: nitrite formation in the absence of HQNO; grey circles: nitrate reduction in the presence of HQNO; green squares: nitrite formation in the presence of HQNO. Formate served as electron donor. HQNO concentration was 320 nmol/mg protein. All measurements were carried out in biological triplicates.

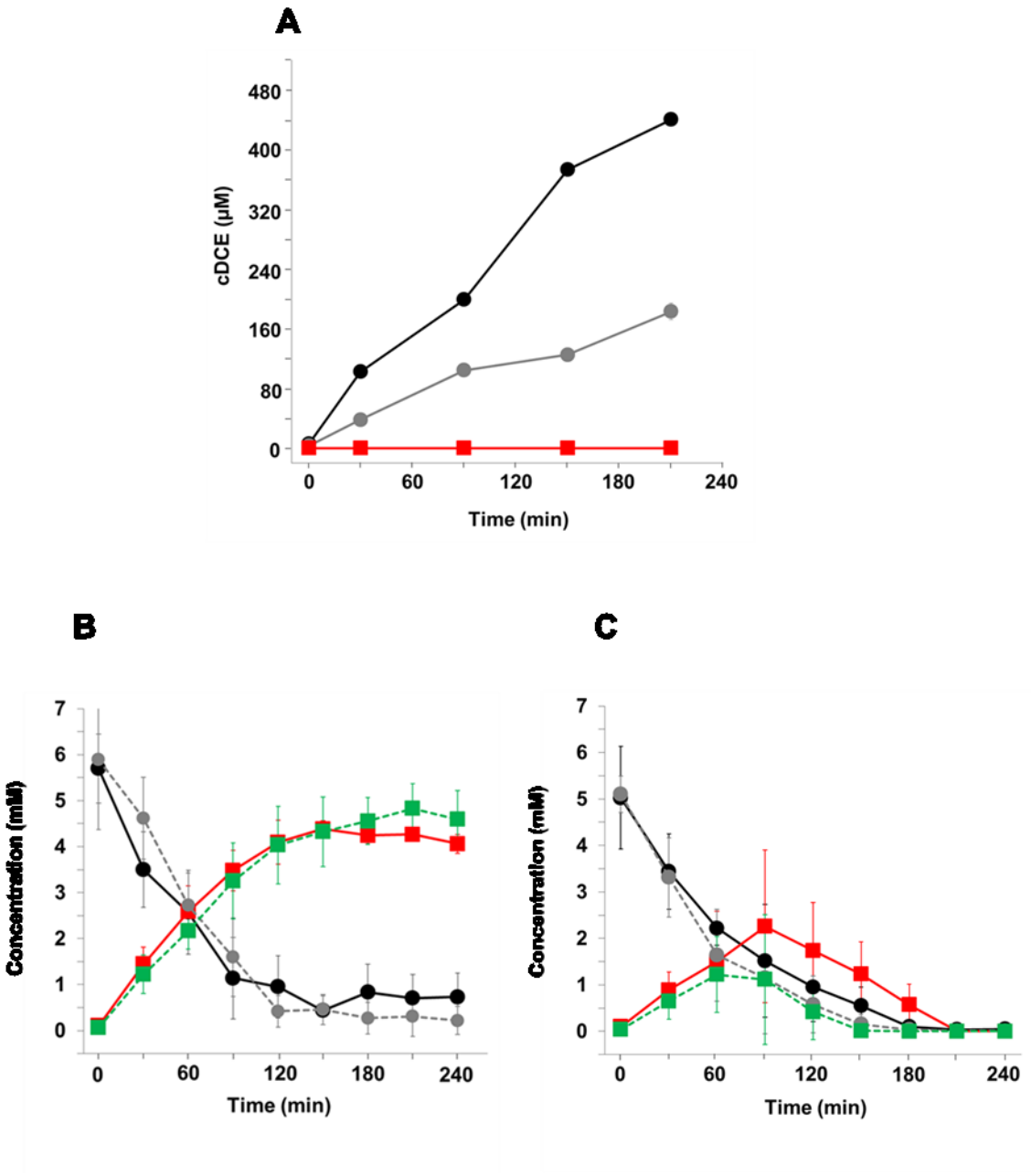

Figure 3: Effect of FCCP on PCE- and nitrate-respiration in cell suspensions of *S. multivorans* and *W. succinogenes*. A: PCE-respiration of *S. multivorans*. Depicted is the formation of cDCE with formate or pyruvate as electron donor in the presence respectively absence of FCCP. Black circles: control without FCCP, pyruvate served as electron donor; grey circles: control without FCCP, formate served as electron donor; red squares: +FCCP, pyruvate respectively formate served as electron donor. **B / C: Nitrate-respiration of *S. multivorans* (B) and *W. succinogenes* (C).** Depicted is the reduction of nitrate (initially 5 mM) and the formation of nitrite with formate as electron donor in the presence respectively absence of FCCP. Black circles: nitrate reduction in the absence of FCCP; red squares: nitrite formation in the absence of FCCP; grey circles: nitrate reduction in the presence of FCCP; green squares: nitrite formation in the presence of FCCP. In all shown experiments FCCP concentration was 15 nmol/mg protein. All measurements were carried out in biological triplicates.

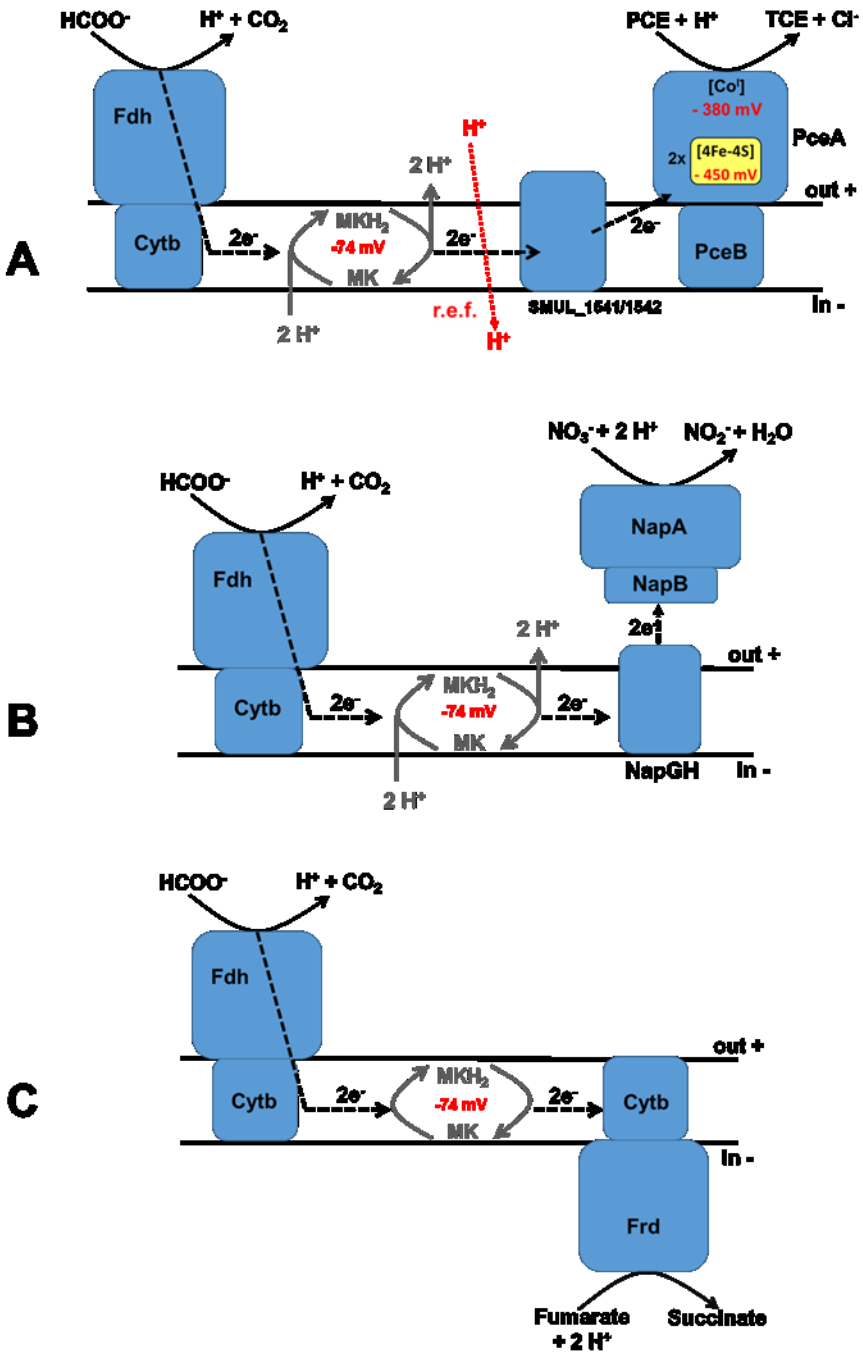

Figure 4: Tentative schemes of three anaerobic respiratory chains of *S. multivorans* using formate as electron donor. Fdh: formate dehydrogenase, integrated in the membrane by its di-heme cytochrome b subunit (FdhC); MK/MKH2: menaquinone/menaquinol. **A: PCE respiratory chain upon reductive dechlorination of PCE**. PceA: reductive dehalogenase, catalytically active subunit; PceB: putative membrane anchor; r.e.f.: reverse electron flow. **B: Nitrate respiratory chain upon nitrate reduction**. NapA: nitrate reductase, catalytically active subunit; NapB: di-heme c-type cytochrome; NapGH: putative quinol dehydrogenase. **C: Fumarate respiratory chain upon fumarate conversion.** Frd: fumarate reductase, integrated in the membrane by its di-heme cytochrome b subunit (FrdC). For detailed information see **Results** and **Discussion** section.

Table 1: Inhibition of different respiratory chains of *S. multivorans* with HQNO and FCCP / CCCP. The results shown in the table are valid for the following inhibitor concentrations, unless otherwise stated: HQNO: 320 nmol/mg protein, 640 nmol/mg protein; FCCP / CCCP: 15 nmol/mg protein, 30 nmol/mg protein. **-**: no inhibition of respiration.

Inhibitor	Electron donor	Electron acceptor	Inhibition of respiratory chain
HQNO	formate	PCE	100%
		nitrate	100%
		fumarate	54% (89%*)
	pyruvate	PCE	100%
		nitrate	100%
		fumarate	-
FCCP/CCCP	formate	PCE	100%
		nitrate	-
		fumarate	-
	pyruvate	PCE	100%
		nitrate	100%
		fumarate	29% (40%**)
	lactate	PCE	100%
		nitrate	-
	hydrogen	PCE	100%
		nitrate	-

* 640 nmol HQNO/mg protein

** 30 nmol FCCP/mg protein / 30 nmol CCCP/mg protein

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Supplemental material to
Investigations on the tetrachloroethene respiratory chain of *Sulfurospirillum multivorans*

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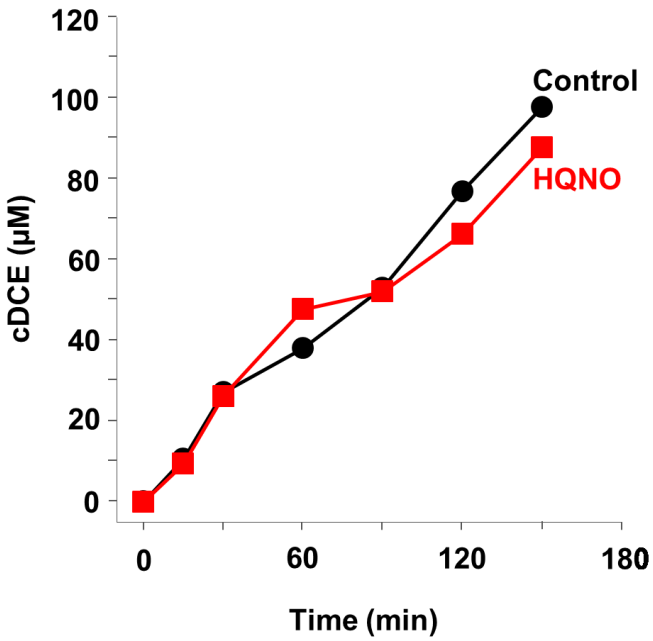

Figure S1: Effect of HQNO (2-n-heptyl-4-hydroxychinolin N-oxide) on PCE-respiration in cell suspensions of *D. hafniense* Y51. Depicted is the formation of cDCE in the presence and absence **(Control)** of HQNO. Pyruvate served as electron donor. Used HQNO concentration was 320 nmol/mg protein. All measurements were carried out in biological triplicates.

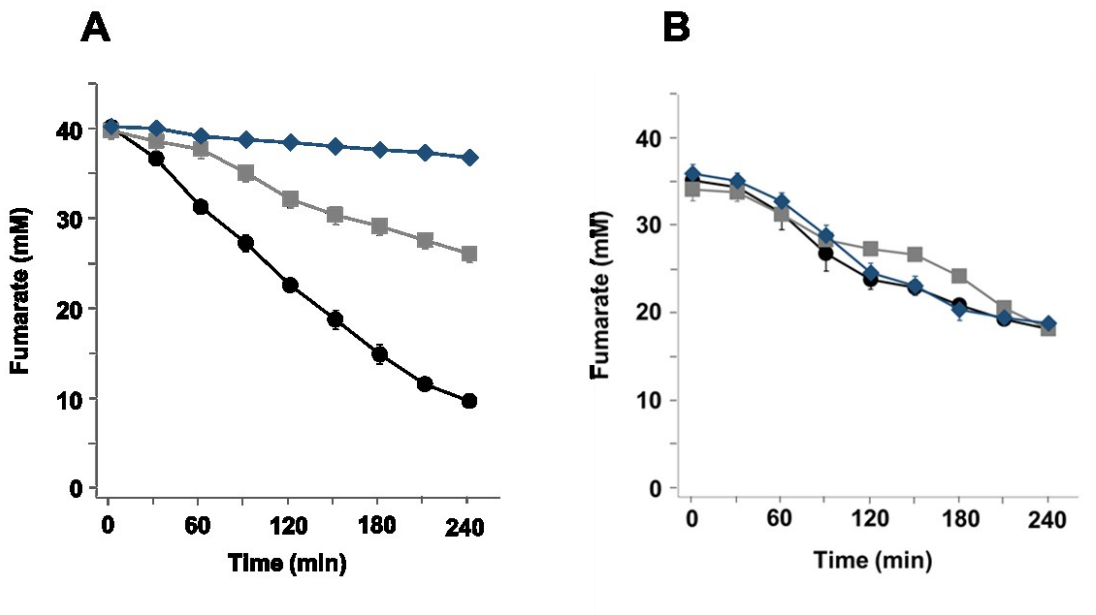

Figure S2: Effect of HQNO (2-n-heptyl-4-hydroxychinolin N-oxide) on fumarate-respiration in cell suspensions of *S. multivorans* and *D. hafniense* Y51. A: Inhibition of fumarate-respiration in *S. multivorans*. Formate served as electron donor. **B:** Inhibition of fumarate-respiration in *D. hafniense* Y51. Pyruvate served as electron donor. Depicted is the conversion of fumarate (initially 40 mM) in the presence and absence of HQNO. Black circles: control without HQNO; grey squares: 320 nmol HQNO/mg protein; blue diamonds: 640 nmol HQNO/mg protein. All measurements were carried out in biological triplicates.

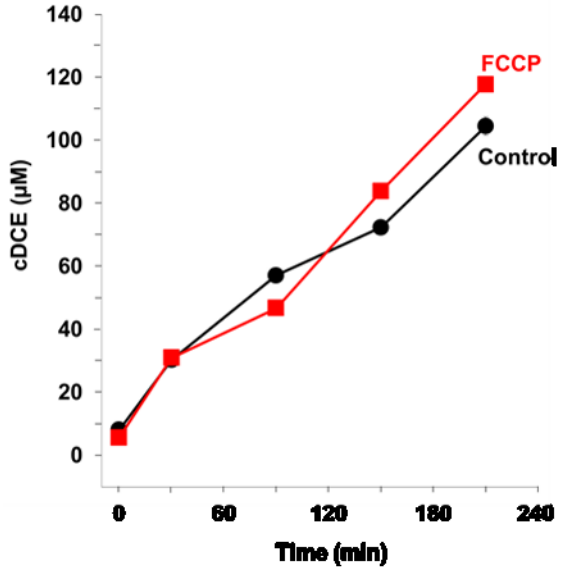

Figure S3: Effect of FCCP (Carbonyl cyanide-p-(trifluoromethoxy)-phenylhydrazone) on PCE-respiration in cell suspensions of *D. hafniense* Y51. Depicted is the formation of cDCE in the presence and absence **(Control)** of FCCP. Pyruvate served as electron donor. Used FCCP concentration was 15 nmol/mg protein. All measurements were carried out in biological triplicates.

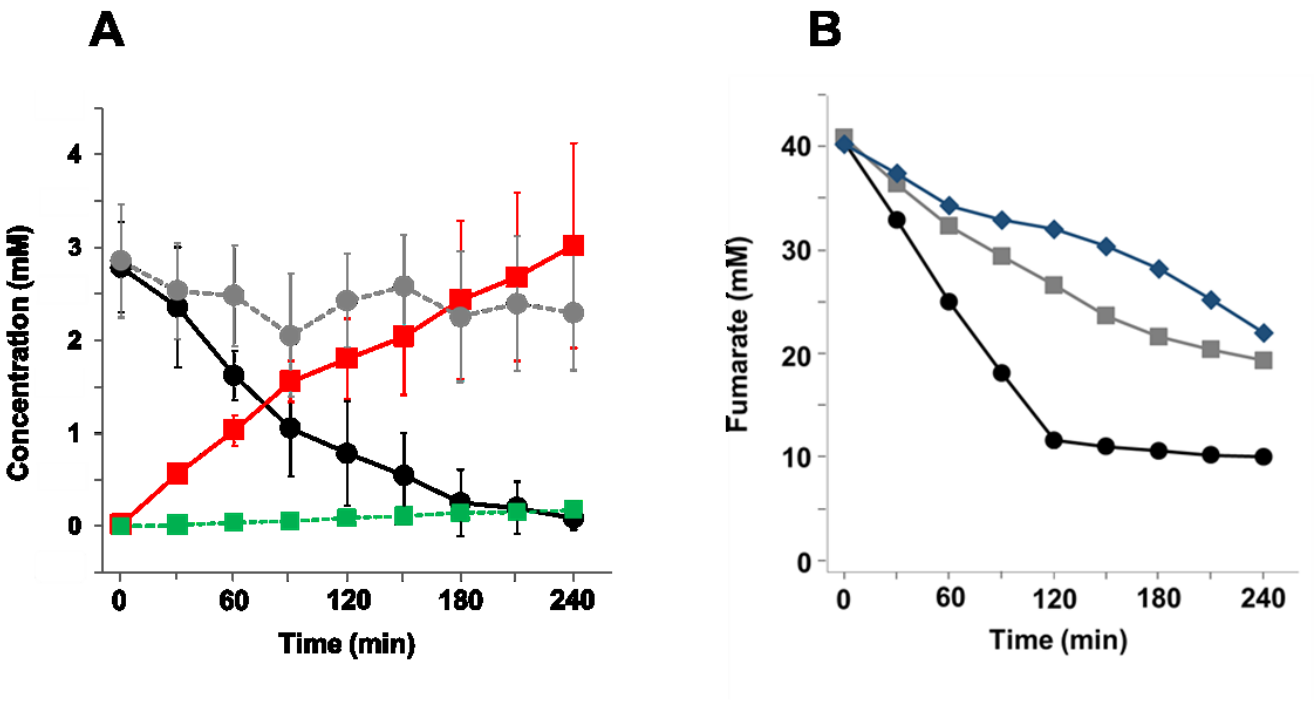

Figure S4: Effect of FCCP (Carbonyl cyanide-p-(trifluoromethoxy)-phenylhydrazone) on nitrate- and fumarate respiration in cell suspensions of *S. multivorans* with pyruvate as electron donor. A: Inhibition of nitrate-respiration. Depicted is the reduction of nitrate (initially 5 mM) and formation of nitrite. Used FCCP concentration was 15 nmol/mg protein. Black circles: nitrate reduction in the absence of FCCP; red squares: nitrite formation in the absence of FCCP; grey circles: nitrate reduction in the presence of FCCP; green squares: nitrite formation in the presence of FCCP. **B:** Inhibition of fumarate-respiration. Depicted is the conversion of fumarate (initially 40 mM). Black circles: control without FCCP; grey squares: 15 nmol FCCP/mg protein; blue diamonds: 30 nmol FCCP/mg protein. All measurements were carried out in biological triplicates.

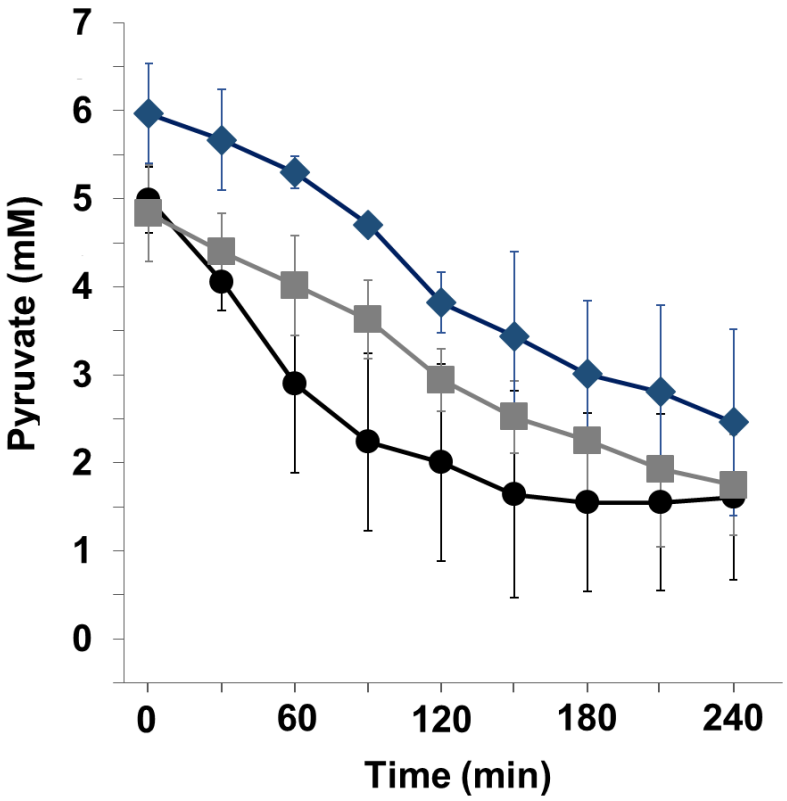

Figure S5: Effect of FCCP (Carbonyl cyanide-p-(trifluoromethoxy)-phenylhydrazone) on pyruvate fermentation in cell suspensions of *S. multivorans*. Depicted is the conversion of pyruvate (initially 5 mM) in the presence and absence of FCCP. Black circles: control without FCCP; grey squares: 15 nmol FCCP/mg protein; blue diamonds: 30 nmol FCCP/mg protein. All measurements were carried out in biological triplicates.

Table S1: Inhibition of different respiratory chains of *Desulfitobacterium hafniense* Y51, *Sulfurospirillum halorespirans*, *Sulfurospirillum deleyianum* and *Wolinella succinogenes* with HQNO and FCCP / CCCP. The results shown in the table are valid for the following inhibitor concentrations, unless otherwise stated: HQNO: 320 nmol/mg protein, 640 nmol/mg protein; FCCP / CCCP: 15 nmol/mg protein, 30 nmol/mg protein. **-**: no inhibition of respiration.

Inhibitor	Organism	Electron donor	Electron acceptor	Inhibition of respiratory chain
HQNO	*Desulfitobacterium hafniense* Y51	pyruvate	PCE	**-**
			fumarate	**-**
	Wolinella succinogenes	formate	nitrate	100%
FCCP/CCCP	*Desulfitobacterium hafniense* Y51	pyruvate	PCE	**-**
	Sulfurospirillum halorespirans	formate	PCE	100%
		formate / hydrogen /lactate	nitrate	**-**
			fumarate	**-**
		pyruvate	nitrate	100%
			fumarate	35% (67%*)
	Sulfurospirillum deleyianum	formate / hydrogen /lactate	nitrate	**-**
			fumarate	**-**
		pyruvate	nitrate	100%
			fumarate	33% (54%*)
	Wolinella succinogenes	formate	nitrate	**-**

* 30 nmol FCCP/mg Protein / 30 nmol CCCP/mg protein

3.5 Manuskript V

Purification of the periplasmatic component of a putative quinol dehydrogenase involved in tetrachloroethene respiration in *Sulfurospirillum multivorans*

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Abstract

The Epsilonproteobacterium *Sulfurospirillum multivorans* is able to grow by Tetrachloroethene (PCE) respiration. Recently it was shown that a NapGH-like quinol dehydrogenase might be a component involved in the electron transfer within the PCE respiratory chain of *S. multivorans*. In this study the periplasmic component (SMUL_1541) of this putative quinol dehydrogenase was heterologously (as C-terminal fusion with a Strep-tag) expressed in *Escherichia coli* and purified by affinity chromatography. After optimization of the growth and induction conditions of *E. coli* as well as after reconstitution of the purified protein (SMUL_1541_{Strep}) with Fe/S clusters we received a SMUL_1541_{Strep} which contained up to 8 mol iron per mol enzyme, what represents 50% of the predicted iron content.

Introduction

A special type of anaerobic respiration, performed by phylogenetically diverse bacteria, is organohalide respiration (OHR), in which reductive dehalogenation of organic halogenated compounds to energy conservation is coupled (Leys *et al.*, 2013). The bacteria able to grow by organohalide respiration can be classified in strict organohalide respiring bacteria (OHRB) (*Dehalobacter* and *Dehalococcoides*), which are restricted to organohalide respiration for energy conservation and the versatile OHRB, which can also grow with other substrates (*Desulfitobacterium* and *Sulfurospirillum*) (Atashgahi *et al.*, 2016). The key enzymes of organohalide respiration are the reductive dehalogenases (RdhA), which display conserved features, such as a Tat (twin-arginine translocation) signal peptide, two Fe/S clusters, and the presence of a corrinoid cofactor at the active site (Schubert & Diekert, 2016).

The gram-negative ε-Proteobacteria *Sulfurospirillum multivorans*, which is a versatile OHRB, is able to grow by reductive dechlorination of the environmental pollutant tetrachloroethene (PCE) using different electron donors like hydrogen, formate or pyruvate (Goris & Diekert, 2016; Scholz-Muramatsu *et al.*, 1995). Though PCE reductive dehalogenase of *S. multivorans*, PceA, is well characterized (Neumann *et al.*, 1996; Bommer *et al.*, 2014), the electron transport chain, responsible for the electron transfer between the electron donor and the reductive dehalogenase, is not elucidated until now. By analyzing the genome and proteome of *S. multivorans*, two proteins probably involved in the PCE respiratory chain were identified, (Goris *et al.*, 2014; Goris *et al.*, 2015). The genes coding for these two proteins are SMUL_1541 and SMUL_1542, encoded 10 respectively 11 genes downstream of PceA. For both gene products the same transcriptional

regulation as for the reductive dehalogenase PceA was observed. In the absence of PCE as a terminal electron acceptor during several transfers of *S. multivorans*, the expression of *pceA* decreased gradually, which could likewise be determined for the putative quinol dehydrogenase genes as well as the genes of the entire OHR core region (John *et al.*, 2009; Goris *et al.*, 2014; Goris *et al.*, 2015). The gene products of SMUL_1541/1542 show similarities to the putative quinol dehydrogenase NapGH of *Wolinella succinogenes*, involved in the electron transfer from the menaquinone pool to the terminal reductase in nitrate respiration, the periplasmic NapA (Kern & Simon, 2008). The NapG-like protein encoded by SMUL_1541 contains four [4Fe-4S] cluster binding motifs and an N-terminal Tat signal peptide. Therefore the protein is considered to be localized in the periplasm while the NapH homologue – SMUL_1542 – harboring two [4Fe-4S] cluster binding motifs is predicted to contain four membrane-spanning helices when predicted to topology prediction (Goris *et al.*, 2014). The only other putative quinol dehydrogenase which might be involved in OHR is encoded in *Desulfomonile tiedjei*, a 3-chlorobenzoate-respiring δ-Proteobacterium. The putative quinol dehydrogenases of *S. multivorans* and *D. tiedjei* form a distinct clade in the phylogenetic tree of quinol dehydrogenases (Goris *et al.*, 2014).

To learn more about the putative quinol dehydrogenase and its possible involvement in the PCE respiration of *S. multivorans* the periplasmic subunit of the putative quinol dehydrogenase – SMUL_1541 - should be purified and characterized. Since *S. multivorans* is currently not genetically accessible, the production of the protein as C-terminal fusion with Strep-tag was performed in *Escherichia coli*.

Materials and Methods

Cultivation of *Sulfurospirillum multivorans* and isolation of genomic DNA

Sulfurospirillum multivorans was cultivated as described previously (Scholz-Muramatsu *et al.*, 1995). The cells were grown with 40 mM pyruvate and 40 mM fumarate. The cells were harvested by centrifugation for 10 min at 12,000 x g and at 10°C. Isolation of genomic DNA was carried out by phenol-chloroform extraction (Bollet *et al.*, 1991).

Cloning of the periplasmatic component of the putative quinol dehydrogenase SMUL_1541

The gene *SMUL_1541* was cloned as a fusion protein with a C-terminal Strep-tag II using the pASK-IBA63c-plus expression vector (IBA GmbH, Göttingen, Germany). The insert was obtained in the following reaction mixture: 50 ng of DNA, 2.5 pmol each primer, 60 mM Tris-HCl (pH 8.5), 25 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 10 mM 2-mercaptoethanol, 200 µM deoxynucleoside triphosphates (dNTPs), and 2.5 U of Taq DNA polymerase (Segenetic, Borken, Germany) in a final volume of 50 ml. After an initial denaturation step of 2 min at 95°C, 35 cycles of 1 min at 95°C, 30 s at 50°C, and 2 min at 72°C were performed. After the last cycle, a final elongation step of 10 min at 72°C was performed. The following primer (5' → 3') were used: *SMUL_1541* (forward): ATATCCATGGATTTTAATCGAAG and *SMUL_1541* (revers): CCAAAGCGCTATTTGATTCATTTTTTG. PCR product and pASK-IBA63c-plus were digested with AfeI and NcoI according to the manufacturer's protocol. The ligation was performed at 16°C overnight in a solution containing 40mMTris-HCl (pH 7.8), 10mM $MgCl_2$, 10mM dithiothreitol (DTT), 0.5 mM ATP, and 5 U of T4 ligase in a final volume of 30 µl. The reaction was stopped by heat inactivation at 65°C for 10 min. The plasmids were transformed in *Escherichia coli* DH5α by heat shock and were recovered from transformants using GeneJET plasmid miniprep kit (Fermentas GmbH, St. Leon-Rot, Germany). Plasmids containing the insert were then transformed to *E. coli* expression strains: *E. coli* BL21 (DE3) and E. coli BL21 (DE3) ΔiscR.

Heterologous expression of the *pceG* gene in Escherichia coli.

E. coli strains BL21(DE3) was grown aerobically in LB medium (5 g yeast extract, 10 g tryptone, 10 g NaCl per liter) supplemented with ampicillin (100 mg/L). Gene expression was induced when the cultures reached an optical density (OD_{578}) of 0.4 -0.5 by adding anhydrotetracycline (AHT; 100 ng/mL).

E. coli BL21 (DE3) ΔiscR was cultivated and heterologous gene expression induced as described in Kuchenreuther *et al.* (2010). *E. coli* BL21 (DE3) ΔiscR was grown in LB medium containing

kanamycin (25 mg/L), ampicillin (100 mg/L), 0.5% w/v glucose (25 mM) and 100 mM MOPS/NaOH (final pH of medium was 7.5). The ΔiscR strain contains a chromosomal substitution of the iscR gene with another gene conferring resistance to kanamycin (Akhtar & Jones, 2008). Initially, all cultures were grown aerobically until an OD_{578} of 0.4–0.5. They were then sealed and anaerobized by alternate evacuating and flushing with N_2. While ferric ammonium citrate (2 mM) was added to the growth medium prior to inoculation, both cysteine (2 mM) and fumarate (25 mM) were added with AHT (100 ng/ml) after the cultures were anaerobized. 100 mL cultures were grown for investigating the induction of heterologous expression, while 1 – 3 L cultures were grown for hydrogenase purification work. *E. coli* was grown at 18°C, 24°C or 28°C as required for the respective experiment. After induction, the cells were harvested by centrifugation for 10 min at 12,000 x g and stored at -20°C.

Purification of heterologous expressed protein SMUL_1541-Strep

The recombinant protein was purified by affinity chromatography on Strep-Tactin. All steps of the enzyme purification where conducted under anoxic conditions. The cells were disrupted using a French Press (1000 psi). The particulate fraction was separated by ultracentrifugation (260,000 x g, 45 min at 4°C) and the resulting supernatant was decanted (supernatant 1 = soluble fraction, Figure 1). The SMUL_1541-Strep was purified from the soluble fraction via gravity flow using the Strep-Tactin Superflow column material (IBA, Göttingen, Germany). The Strep-Tactin column was equilibrated with buffer A (100 mM Tris-HCl pH 8.0), which was also used for washing the column after the cell extract has entered the column material. SMUL_1541-Strep was eluted from the column material with buffer B (100 mM Tris-HCl pH 8.0, 2.5 mM desthiobiotin). SMUL_1541-Strep was solubilized from the particulate fraction by stirring the particulate fraction overnight at 4°C in either buffer A or detergent solutions (0.5% w/v Digitonin, 1.0% w/v Triton X100 or 1.0% w/v N-laurylsarcosine in 100 mM Tris-HCl pH 8.0). After ultracentrifugation (260,000 x g, 45 min at 4°C) the resulting supernatant (supernatant 2, Figure 1) was decanted and SMUL_1541-Strep purified as described for the soluble fraction. SMUL_1541-Strep was concentrated using a Vivaspin 6 (10 KDa) ultrafiltration unit (Sartorius, Göttingen, Germany). SMUL_1541-Strep was transferred into small anoxic rubber-stoppered glass vials (0% oxygen) and stored at 4°C respectively at -20°C for longer times. Homogeneity of the purified protein was determined via SDS-PAGE and immunoblot analysis.

Test for recombinant protein production using SDS-PAGE

At the times indicated, 5-ml samples were taken from *E. coli* cultures. After centrifugation (12,000 x g, 10 min at 10°C), the cell pellets were stored at -20°C. The pellets were suspended in 2.5 ml buffer (100 mM Tris-HCl pH 7.5) and cells were disrupted using a French-Press (1000 psi). Particulate fraction was separated by ultracentrifugation (260,000 x g, 45 min at 4°C) and the resulting supernatant (soluble protein) decanted. The obtained particulate fraction was suspended in 2 ml Tris-HCl buffer. The supernatant as well as the particulate fraction were used for protein determination (see "Analytical methods" below). SDS-PAGE was performed according to a method described previously by Laemmli (1970), using a Tris-glycine-SDS buffer system. Total protein (crude extract) was sampled directly after cell disruption.

Immunoblot analysis

Protein samples were subjected to denaturing SDS PAGE (12.5%) and afterwards blotted onto a polyvinylidene difluoride (PVDF) membrane (Roche, Mannheim, Germany) using a semi-dry transfer cell (Bio-Rad, Munich, Germany) according to the protocol described by John *et al.* (2009). SMUL_1541-Strep was detected using a specific antibody against the Strep-Tag. The primary antibody was detected via a secondary antibody (diluted 1:20,000) coupled to alkaline phosphatase (Sigma-Aldrich, Munich, Germany).

In vitro reconstitution of [Fe-S] clusters

The gas atmosphere of the purified SMUL_1541-Strep protein solution was replaced by nitrogen. For reconstitution of [Fe-S] clusters, 10 mM DTT and a 10-fold molar excess of NH_4-Fe(III)-citrate and Li_2S were added. The solution was incubated for 16 h at 4°C under N_2 as the gas phase. To remove unbound iron and sulfide, the protein sample was washed - 100 mM Tris-HCl buffer, pH 8.0 - and concentrated using a Vivaspin 6 (10 kDa) ultrafiltration unit (Sartorius, Göttingen, Germany). All used solutions were anoxic.

UV-visible absorption spectra were recorded on a Cary 300 Bio UV/VIS Spectrophotometer (Varian, Darmstadt, Germany). The used cuvettes were sealed and flushed with nitrogen.

Analytical methods

Protein concentrations were determined in accordance to the method described by Bradford (1976) using the Bio-Rad reagent (Bio-Rad Laboratories, Munich, Germany). Bovine Serum Albumin was used as standard. Iron was quantified using the method of Fish (1988).

Identification of proteins via Mass spectrometry and proteome data analysis

Purified protein was subjected to SDS-PAGE. Afterwards the obtained protein bands were cut out and prepared for proteolytic cleavage using trypsin (Promega, Madison, WI, USA). Peptide lysates were extracted and desalted using C18 Zip Tips (Merck Millipore, Darmstadt, Germany). The mass spectrometry analysis was performed on an Orbitrap Fusion mass spectrometer (Thermo Scientific, San Jose, CA, USA). MS data were analyzed using Proteome Discoverer (v1.4.1.14, Thermo Scientific). MS spectra were searched against the data of the *S. multivorans* database containing 3,191 non-redundant protein-coding sequence entries (downloaded February 17th 2014 from NCBI Genbank accession number CP007201) and the data of the *Escherichia coli* BL21-Gold (DE3)pLysS AG database (downloaded April 11th 2014 from NCBI Genbank accession number CP001665). False discovery rate for peptides was 1%.

Results

Purification of SMUL_1541-Strep from *E. coli* BL21 (DE3)

C-terminally strep-tagged SMUL_1541 was purified by affinity chromatography out of an *E. coli* (3L-culture) harboring pASK-IBA63c-plus-derived plasmid containing the coding region of SMUL_1541. *E. coli* was induced at an optical density of 0.5 with anhydrotetracycline and cultivated for another 24 hours on LB medium at 28°C under shaking. The purification was performed under anoxic conditions by using the soluble fraction as depicted in Figure 1.

The apparent molecular mass of the purified protein was determined via immunoblot analysis using a specific antibody against the Strep-Tag. The detected apparent molecular mass was identical to the theoretical molecular mass calculated for SMUL_1541-Strep (24.68 kDa) (Figure 2B). The purity of SMUL_1541 was determined via SDS-PAGE and Coomassie staining (Figure 2A). Besides a band correlating with the molecular mass of SMUL_1541-Strep, two more bands were detected. All three bands were cut out and subjected to mass spectrometric analysis for protein identification (see Materials and Methods). The 24.68 kDa large protein was identified as SMUL_1541$_{Strep}$. The other proteins were identified as DnaK and GroEL, chaperons of the Hsp70- respectively Hsp60-family (Hsp: Heat Shock Protein).

The amount of 980 µg protein could be purified. Looking at the Coomassie stained SDS-PAGE GroEL seemed to represent the major amount of protein (Figure 2A). The enriched protein was nearly colorless suggesting an uncomplete occupation of SMUL_1541$_{Strep}$ with [Fe-S] clusters.

Optimization of the purification of SMUL_1541$_{Strep}$

The aims of the optimization were to increase the protein yield, to purify homogenous protein and to reconstitute SMUL_1541$_{Strep}$ with [Fe-S] clusters.
Therefore, the expression strain *E. coli* BL21 (DE3) ΔiscR, specifically engineered for improved synthesis of [Fe-S] cluster containing proteins was used (Akhtar & Jones, 2008). Growth and induction conditions were changed as follows. For *E. coli* BL21 (DE3) ΔiscR growth and induction conditions were chosen as described in Kuchenreuther *et al*., 2010 (see Materials and Methods). The induction as well as the following synthesis of the recombinant proteins were performed under anoxic conditions. Glucose and the electron acceptor fumarate were added to the medium to stimulate the anaerobic metabolism and to allow the growth under these conditions. Furthermore, iron and cysteine was added to the medium.

Induction of SMUL_1541-Strep in E. coli BL21 (DE3) ΔiscR

E. coli BL21 (DE3) ΔiscR was cultivated under the conditions described in Kuchenreuther *at al.*, 2010 (see Materials and Methods) at 18°C and 24°C. Cultures were induced at an optical density of 0.5 and samples were taken at defined time points after induction of SMUL_1541$_{Strep}$ production. For both temperatures tested the same SMUL_1541$_{Strep}$ induction pattern could be observed. The following information refer to cells of *E. coli BL21* (DE3) ΔiscR which were induced and grown at 24°C. In the time period between one and two hours after induction a significant increase of SMUL_1541$_{Strep}$ amount could be observed (Figure 3) while afterwards no other significant increase in protein yield could be detected (data not shown). The induction time had no influence on the occupation of SMUL_1541$_{Strep}$ with [Fe-S] clusters. An iron content of 1 – 2 mol Fe/mol SMUL_1541$_{Strep}$ could be determined for SMUL_1541$_{Strep}$ purified from cells harvested 1.5 hours as well as from cells harvested 24 hours after induction. The major amount of SMUL_1541$_{Strep}$ could be detected in the particulate fraction, which consisted of cell debris and membrane, at any investigated time point after induction of protein synthesis (Figure 3).

Because of these results cells grown and induced at 24°C and harvested 1.5 hours after induction of SMUL_1541$_{Strep}$ production were used for all protein purifications described in the following. For the described purifications always a 3L-cultures of *E. coil* BL21 (DE3) Δ*iscR* (about 6 g) was used.

Purification of SMUL_1541$_{Strep}$ from *E. coli* BL21 (DE3) ΔiscR

SMUL_1541$_{Strep}$ was purified from the soluble fraction (supernatant 1) and the particulate fraction (supernatant 2) as depicted in Figure 1. In this case no detergent was used for the solubilization of SMUL_1541$_{Strep}$ out of the particulate fraction but by stirring the particulate fraction in washing buffer (100 mM Tris-HCl, pH 8.0) overnight (see Materials and Methods).

1000 µg protein and 398 µg protein were purified from the soluble fraction respectively particulate fraction (Figure 4). A big amount of SMUL_1541$_{Strep}$ seemed to be associated with the particulate fraction as it was already observed in the induction experiment (see above). By washing the particulate fraction overnight, no significant amounts of SMUL_1541$_{Strep}$ could be obtained from this fraction. The purified protein fractions were almost colorless what suggested an incomplete occupation of SMUL_1541$_{Strep}$ with [Fe-S] clusters.

From the soluble fraction of *E. coli* BL21 (DE3) Δ*iscR*, grown and induced as described in Kuchenreuther *et al.*, 2010, similar amount of SMUL_1541$_{Strep}$ (around 1000 µg) could be purified as from the soluble fraction of *E. coli* BL21 (DE3) grown on LB medium (see above). In both

purifications a large amount of the purified protein seemed to be the chaperon GroEL (compare Figure 2A and Figure 4A).

Usage of detergent by the purification of SMUL_1541$_{Strep}$ from the particulate fraction of *E. coli* BL21 (DE3) ΔiscR

SMUL_1541$_{Strep}$ shows a strong association with the membrane, possibly attributed to hydrophobic interactions. Therefore, the yield of purified SMUL1541$_{Strep}$ from the particulate fraction should be increased by the usage of detergent. Digitonin (0.5%), Triton X100 (1%) and N-laurylsarcosine (1%) were tested.

Using Digitonin and Triton X100, 400 µg respectively 72 µg protein were purified from the particulate fraction. Besides SMUL_1541$_{Strep}$ also GroEL was purified. Because of the results both detergents were not qualified for the purification of SMUL_1541$_{Strep}$ from the particulate fraction. When using N-laurylsarcosine, the yield of purified protein could be increased significantly. The comparison of different purifications showed that about 1700 µg protein were purified from the particulate fraction of *E. coli* BL21 (DE3) ΔiscR. The proportion of GroEL on purified protein was significantly reduced. Almost the total purified protein was SMUL_1541$_{Strep}$ (Figure 5). The obtained protein showed an obvious yellowish brown color. This coloring was interpreted as occupation of SMUL_1541$_{Strep}$ with [Fe-S] cluster.

Reconstitution of SMUL_1541$_{Strep}$ with [Fe-S] clusters

The periplasmic component of the putative quinol dehydrogenase SMUL_1541 is predicted to harbor four [4Fe-4S] clusters. Thus, a complete occupation of SMUL_1541 with [Fe-S] clusters should result in 16 mol Fe per mol protein. The iron content of the obtained protein was measured. For the investigation SMUL_1541$_{Strep}$, purified from the particulate fraction of *E. coli* BL21 (DE3) ΔiscR by utilizing N-laurylsarcosine, was used.

Purified SMUL_1541$_{Strep}$ harbored up to 2 mol Fe per mol protein, representing 12.5% of the predicted iron content. To improve the [Fe-S] clusters content, the protein was reconstituted. The successful reconstitution of [4Fe-4S] clusters was confirmed either by the deep brownish color of the protein solution as well as by the UV-visible spectrum absorption band at 410 nm (Figure 6). The reduction of [Fe-S] clusters with Titan(III)citrate, causing a decrease of absorption at 410 nm, as well as the incomplete re-oxidation of the clusters by incubation with air was possible (Figure 6).

After reconstitution of the [Fe-S] clusters SMUL_1541$_{Strep}$ harbored up to 8 mol Fe per mol protein, what represented 50% of the predicted iron content.

Conclusions

Optimization of the growth and induction conditions of *E. coli* BL21 (DE3) Δ*iscR* led to a purified and reconstituted SMUL_1541$_{Strep}$ which contained up to 8 mol iron per mol enzyme, what represents 50% of the predicted iron content. In view of future experiments, the aim should be to achieve a complete occupation of the enzyme with iron sulfur clusters.

A marked reduction in the *E. coli* cultivation temperature from 24°C to temperatures between 4 and 10°C after the induction of SMUL_1541 production might favor an occupation of the enzyme with [Fe-S] clusters. A lower cultivation temperature leads to a slower production of the protein and might therefore result in a longer time for the formation and incorporation of iron-sulfur clusters.

In this study the chaperons DnaK and specially GroEL were always co-purified with SMUL_1541$_{Strep}$. Both proteins are representatives from two of the three chaperone systems responsible for the folding of newly synthesized proteins in *E. coli*: DnaK-DnaJ-GrpE and GroEL-GroES (Baneyx & Mujacic, 2004). These proteins are maybe involved in the folding of SMUL_1541$_{Strep}$ in *E. coli*. The chaperons GroEL and DnaK can also be found in *S. multivorans* (Goris *et al.*, 2015). Therefore the co-expression of SMUL_1541 with either GroEL respectively DnaK from *E. coli* or from *S. multivorans* might be worth a try. For various iron-sulfur proteins it has already been shown that overexpression of *isc* leads to an improved occupation of these proteins with iron-sulfur clusters (Kriek *et al.*, 2003; Kuchenreuther *et al.*, 2010). Though until now the exact mechanism of cluster assembly by isc is not fully understood, some important proteins, including IscS, IscU, IscA, HscB, HscA and Fdx, have been identified (Kriek *et al.*, 2003). In this study we used an *E. coli* mutant insufficient in producing the protein IscR, which is the repressor of the *isc* operon (Schwartz *et al.*, 2001). So, in this mutant the overexpression of the isc operon is constantly induced. We assume that a co-expression of SMUL_1541 with the proteins, involved in [Fe-S] cluster assembly, would not led to other results as we have obtained by using *E. coli* BL21(DE3) ΔiscR. However, in the genomic region of S. *multivorans* in which the SMUL_1541 gene is localized also a gene (SMUL_1533) coding for a small protein, which shows similarity to proteins involved in iron-sulfur cluster maturation (21% amino acid sequence identity to the characterized *E. coli* IscU), is found (Goris *et al.*, 2014; Goris *et al.*, 2015). The protein is predicted to be involved in the correct insertion of the iron-sulfur clusters into the reductive dehalogenase

PceA and might fulfill the same function for SMUL_1541. A co-expression of the protein SMUL_1541 with SMUL_1533 could therefore lead to a better occupation of SMUL_1541 with iron-sulfur clusters.

Funding: This work was supported by the German Research Foundation (DFG), as part of the research unit FOR 1530. The authors used the analytical facilities of the Centre for Chemical Microscopy (ProVIS) at the Helmholtz Centre for Environmental Research, which is supported by European Regional Development Funds (EFRE - Europe funds Saxony) and the Helmholtz Association.

Acknowledgement: Christian L. Schiffmann is thankfully acknowledged for protein identification by mass-spectrometrical analysis.

Main Figures

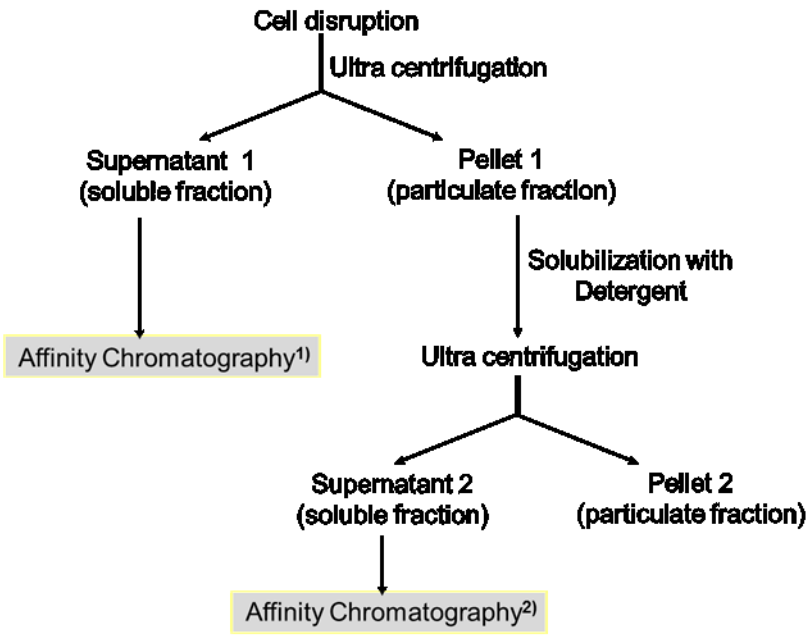

Figure 1: Scheme of purification of SMUL_1541-Strep by affinity chromatography on Strep-Tactin.

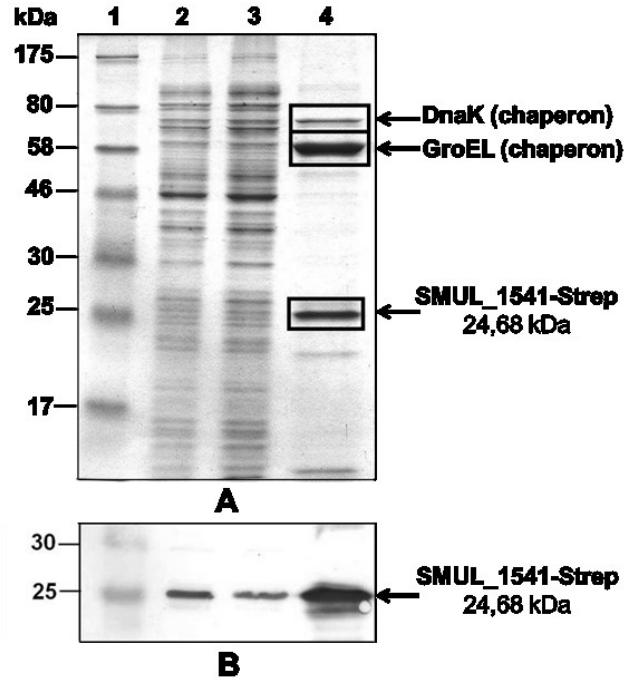

Figure 2: Purification of SMUL_1541-Strep out of *E. coli* BL21 (DE3). A: Coomassie stained SDS-PAGE. **B:** Immunoblot analysis. A specific antibody against the Strep-tag was used for the detection of SMUL_1541-Strep. The marked band were cut out and the proteins identified via MS analysis. Crude extract (lane 2; 10 µg) and soluble fraction (lane 3; 10 mg) of *E. coli* BL21 (DE3) as well as purified protein (lane 4; 1 µg) were loaded. Lane 1 = protein marker.

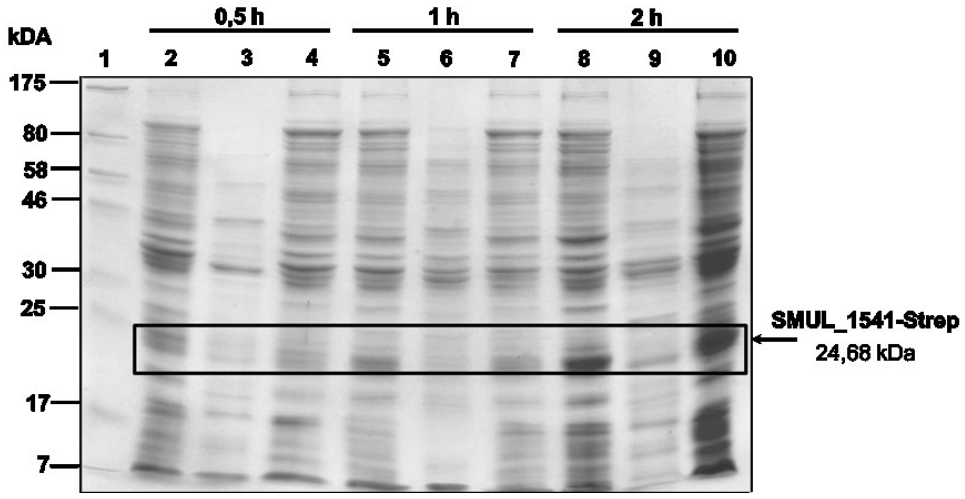

Figure 3: Induction experiment. Coomassie-stained SDS-PAGE for the determination of SMUL_1541$_{Strep}$ production in E. *coli* BL21 (DE3) Δ*iscR* after induction with anhydrotetracycline. The cultivation and induction were performed as described in Kuchenreuther *et al.*, 2010. Samples were taken 0.5, 1 and 2 hours after induction of protein synthesis. 10 µg protein was loaded per lane. The crude extracts (lane: 2, 5, 8), the soluble fractions (lane: 3, 6, 9) and the particulate fractions (lane: 4, 7, 10) were loaded. Lane 1 = protein marker.

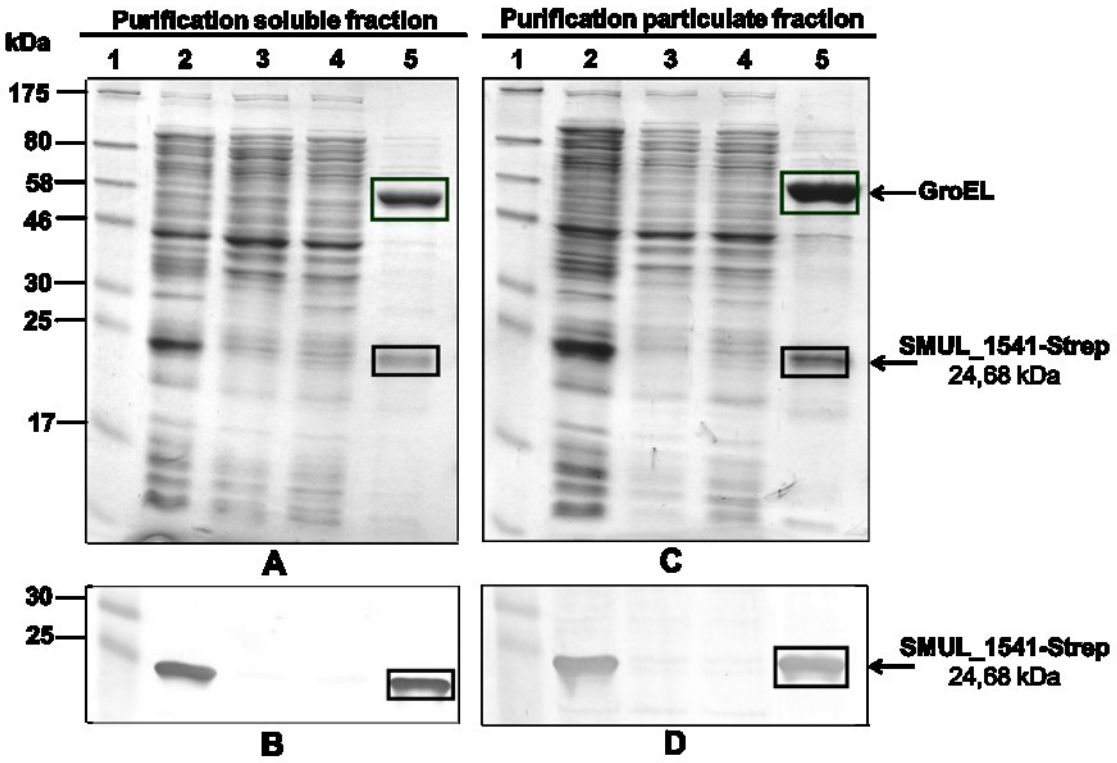

Figure 4: Purification of SMUL_1541$_{Strep}$ from the soluble (supernatant 1) and particulate (supernatant 1) fraction of *E. coli* BL21 (DE3) ΔiscR. A / C: Coomassie-stained SDS-PAGE. **B / D:** Immunoblot analysis. A specific antibody against the strep-tag was used for the detection of SMUL_1541$_{Strep}$. For the purification of SMUL_1541$_{Strep}$ out of the soluble fraction 10 µg protein from the crude extract (lane: 2), soluble fraction (lane: 3) and flow through (lane: 4) as well as 1 µg of the purified protein (lane: 5) were loaded. Lane 1 = protein marker. For the purification of SMUL1541$_{Strep}$ out of the particulate fraction 10 µg protein from the crude extract (lane: 2), soluble fraction (lane: 3) and flow through (lane: 4) as well as 1 µg of the purified protein (lane: 5) were loaded. Lane 1 = protein marker.

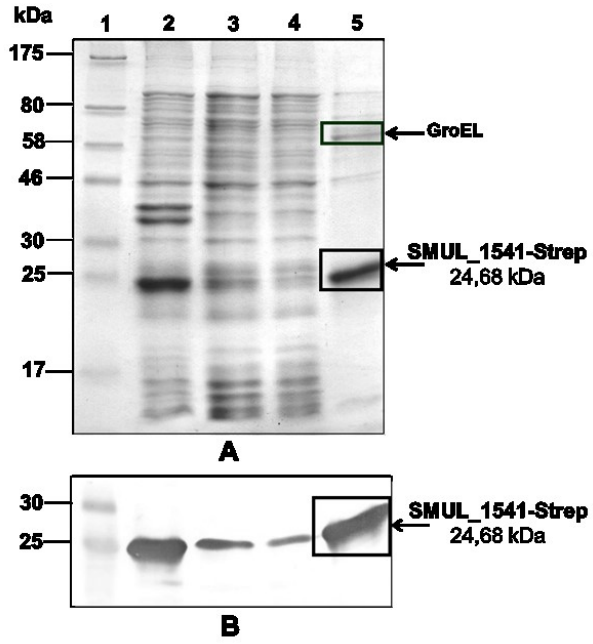

Figure 5: Purification of SMUL_1541$_{Strep}$ from the particulate fraction of *E. coli* BL21 (DE3) ΔiscR using N-laurylsarcosin. A: Coomassie-stained SDS-PAGE. **B:** Immunoblot analysis. A specific antibody against the Strep-tag was used for the detection of SMUL_1541$_{Strep}$. 10 µg protein of the particulate fraction (lane: 2), soluble fraction (supernatant 2; lane: 3) and flow through (lane: 4) as well as 1 µg of the purified protein after affinity chromatography on Strep-Tactin (lane: 5). Lane 1 = protein marker.

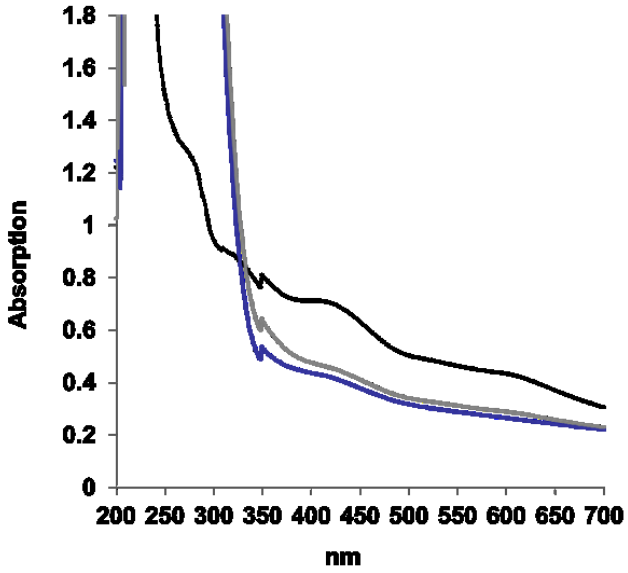

Figure 6: UV-visible spectrum of SMUL_1541$_{Strep}$ after reconstitution of the [Fe-S] clusters. The spectra showed a characteristic absorption band for [4Fe-4S] clusters at 410 nm. Black curve: SMUL_1541$_{Strep}$; blue curve: SMUL_1541$_{Strep}$ + 20 µl Titan(III)citrate; grey curve: SMUL_1541$_{Strep}$ + 20 µl Titan(III)citrate incubated with air.

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3.6 Manuskript VI

Reductive tetrachloroethene dehalogenation in the presence of oxygen by *Sulfurospirillum multivorans*: Physiological studies and proteome analysis

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Abstract

Reductive dehalogenation of organohalides is often carried out by organohalide-respiring bacteria (OHRB) in anoxic environments. The gram-negative, tetrachloroethene (PCE)-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* is one of few OHRB also able to grow with oxygen as terminal electron acceptor. Therefore, we investigated the organism's capacity to reductively dehalogenate PCE in the presence of oxygen, which would broaden the applicability to use OHRB for bioremediation. It is shown that *S. multivorans* performs dehalogenation of PCE to *cis*-1,2-dichloroethene (*c*DCE) at oxygen concentrations below 0.19 mg/ml (0.5%), whereas *Desulfitobacterium hafniense* Y51, an OHRB classified in the Firmicutes, was only able to dehalogenate PCE under strictly anoxic conditions. The redox potential had no influence on reductive dehalogenation by *S. multivorans*, suggesting that higher levels of oxygen impair PCE dechlorination by inhibiting involved enzymes. The PCE reductive dehalogenase remained active in cell extracts of *S. multivorans* exposed to 1% oxygen, so that other enzymes or compounds involved in PCE respiration might be affected. Analysis of the proteome revealed that superoxide reductase and cytochrome peroxidase amounts increased under low oxygen concentrations, while the stress response to atmospheric oxygen concentrations involved catalase and hydrogen peroxide reductase. Taken together, our results demonstrate that OHR is not limited to anoxic environments.

Introduction

Halogenated organic groundwater contaminants (e.g. chlorinated ethenes) are dehalogenated by various aerobic and anaerobic microorganisms (Fetzner, 1998). Aerobic bacteria dehalogenate organohalides via oxidation reactions using different enzymes (van Pée & Unversucht, 2003), whereas anaerobic bacteria often use halogenated compounds as a terminal electron acceptor in an anaerobic respiration in which they couple reductive dehalogenation to energy conservation. This process, termed organohalide respiration (OHR), involves corrinoid-containing reductive dehalogenases as terminal reductase (Leys *et al.*, 2013, Schubert & Diekert, 2016). Organohalide-respiring bacteria (OHRB) are mainly found within the phyla Chloroflexi (*Dehalococcoides* and *Dehalogenimonas*), Firmicutes (*Dehalobacter* and *Desulfitobacterium*), Deltaproteobacteria (*Geobacter*, *Desulfomonile*), and Epsilonproteobacteria (*Sulfurospirillum*) (Atashgahi *et al.*, 2016). Chlorinated ethenes are widely occurring as groundwater contaminants (Moran *et al.*, 2007). While chlorinated ethenes with one or two chlorine substituents (vinyl chloride (VC) and dichloroethene (DCE)) can be dechlorinated oxidatively under oxic conditions (Mattes *et al.*, 2010), tetrachloroethene (PCE) is highly persistent to microbial degradation in the presence of oxygen but is transformed via reductive dechlorination catalyzed by organohalide-respiring bacteria (Atashgahi *et al.*, 2016). Although formerly described as an obligate anaerobic bacterium (Scholz-Muramatsu *et al.*, 1995), *S. multivorans* was recently shown to be able to respire oxygen at low O_2 concentrations (Goris *et al.*, 2014). Besides *S. multivorans* also *Anaeromyxobacter dehalogenans*, a Deltaproteobacterium dechlorinating 2-chlorophenol, is an OHRB tolerating low concentrations of oxygen (Sanford *et al.*, 2002, Thomas *et al.*, 2008). In contrast to *S. multivorans*, *A. dehalogenans* is not capable of PCE dechlorination. A few other aerobic bacteria mainly belonging to the Betaproteobacteria carry out reductive dehalogenation (but not organohalide respiration) under oxic conditions, however, they seem to exclusively dehalogenate brominated aromatic compounds (Chen *et al.*, 2013, Chen *et al.*, 2015).

Microbial dechlorination of PCE has been shown to be performed under anoxic conditions at environmental redox potentials below -180 mV (Kästner, 1991). However, soil and groundwater, which are often contaminated with chlorinated ethenes, are inhomogeneous with respect to their oxygen concentration and include microoxic zones. We here addressed the question if reductive dechlorination could be catalyzed by the facultative microaerobic OHRB *S. multivorans* in the presence of oxygen, since this would enhance the practicability of bioremediation in microoxic contaminated environments (e.g. at oxic/anoxic interphases or microoxic aquifers). Since not much is known about the physiological reaction of microaerobic OHRB or Epsilonproteobacteria

to oxygen, we investigated also the cellular response of *S. multivorans* to oxygen by analyzing the organism's proteome.

Materials and Methods

Cultivation

S. multivorans (DSMZ 12446) was cultivated under anaerobic conditions at 28°C in a defined mineral medium (Scholz-Muramatsu *et al.*, 1995) without vitamin B_{12} (cyanocobalamin). *D. hafniense Y51* was cultivated in the same medium but with 0.2% yeast extract. Pyruvate (40 mM) was used as electron donor, oxygen or PCE as electron acceptor. PCE was added to the medium (10 mM nominal concentration) from a hexadecane stock solution (0.5 M, resulting in a hexadecane to medium ratio of 2%). The oxygen concentration was 5% or 20% in the gas phase of the cultivation bottles. In order to induce long-term down-regulation of genes in the organohalide respiration region (John *et al.*, 2009, Goris *et al.*, 2015), the organism was cultivated for 60 transfers on pyruvate (40 mM) and fumarate (40 mM) plus 0.2% yeast extract. The resulting culture was used as inoculum for all pre-cultures. Pre-cultures were grown in rubber-stoppered 200 mL glass serum bottles with a ratio of gas to liquid phase of 1:1. The third sequential pre-culture was used for inoculation of the main culture. In general, main cultures were grown in rubber-stoppered 2 L glass bottles with a gas to liquid phase ratio of 9:1 when performing experiments with oxygen; when PCE was added as the electron acceptor, the gas to liquid phase ratio was 1:1. Oxygen was added in concentrations from 0 to 20%. Oxygen was introduced into 200 mL glass serum bottles using air-filled syringes. When using 2 L glass bottles, pure oxygen (Linde AG, Leuna, Germany) was added and the cultures were shaken at 150 rpm.

When investigating the oxygen stress response of *S. multivorans*, cells were initially grown anoxically on pyruvate/PCE as described above. In the mid-exponential growth phase, oxygen was added to the gas phase to a final amount of 5% or 20% oxygen, respectively. Cultivation was then continued for 6.5 hours.

For the PCE dechlorination induction experiment, *S. multivorans* was cultivated on pyruvate/PCE (three replicates) and pyruvate/PCE plus 5% oxygen in the gas phase (six main cultures). Three of the latter cultures were harvested when the cultures reached the stationary growth phase, the remaining three cultures after PCE dechlorination had started. Cultures grown on pyruvate/PCE without oxygen were harvested in the exponential growth phase.

Bacterial growth was monitored photometrically by measuring the optical density at 578 nm or by the determination of the protein concentration with the Bio-Rad reagent (Bio-Rad Laboratories, München, Germany) using the method described by (Bradford, 1976). Bovine Serum Albumin was used as standard. All cultivations were performed in triplicates.

First, a pre-culture of *S. multivorans* (100 ml) was harvested under anoxic conditions. The resulting cell pellet was washed twice with an anoxic buffer (100 mM Tris-HCl pH 7.5). Afterwards the cells

were resuspended in 100 ml medium containing pyruvate (40 mM) without PCE. This served as inoculum for the main culture. The bacteria were inoculated in 10 ml pyruvate/PCE medium (see above) in 50 ml Erlenmeyer flasks. The flasks were closed with loose aluminum caps to allow gas exchange. The cultures were cultivated at 28°C under rigorous shaking (360 rpm) in a hypoxic chamber with a nitrogen atmosphere containing 0.2%, 0.5% or 1% oxygen. When the experiment was performed with *Desulfitobacterium hafniense* Y51, the pre-culture as well as the main-culture were cultivated in the presence of yeast extract (0.2%). At defined time points samples were taken for the quantification of chlorinated ethenes. Uninoculated medium-containing flasks treated the same way as the cultures served as a control for abiotic loss of PCE during cultivation.

Cell harvesting and sample preparation

S. multivorans cells were harvested by centrifugation (12,000 x g, 10 min at 10°C). The cell pellets were washed once in 50 mM Tris-HCl (pH 7.5) and resuspended (2 ml per g wet weight) in the same buffer containing a tip of a spatula of DNase I (AppliChem, Darmstadt, Germany) and half a tablet of protease inhibitor (cOmplete Mini, EDTA-free; Roche, Mannheim, Germany). The cells were disrupted using a French Press (6.9 MPas = 1000 psi). Cell debris was removed by centrifugation (6,000 x g, 10 min at 4°C). Protein was precipitated using acetone and 50 µg was takenfrom each sample. Protein pellets were resuspended in 5 x sample buffer (0.31 M Tris-HCl pH 6.8; 10% sodium dodecylsulphate (SDS) (w/v); 30% glycerol (v/v); 25% mercaptoethanol (v/v), 0.05% bromophenol blue (w/v)) and denatured for ten minutes at 66°C. SDS polyacrylamide gel electrophoresis was carried out in a 12.5% acrylamide gel over a running distance of 2 cm. Lanes were cut, destained, and proteins reduced and alkylated as described before (Goris *et al.*, 2016) prior to digest with trypsin (Promega, Madison, WI, USA) overnight at 37°C. Extracted peptides were desalted using ZipTip-µC18 material (Merck Millipore, Darmstadt, Germany). After evaporation of solvents under vacuum, samples were resuspended in 0.1% formic acid containing 2% acetonitrile before LC–MS/MS analysis.

Mass spectrometry and proteome data analysis

MS spectrometric analysis was performed as described previously (Goris *et al.*, 2015, Goris *et al.*, 2016). In short, an Ultimate 3000 nanoRSLC system (Thermo Scientific, Germering, Germany) was used to separate peptides with a 90 min gradient. After electron spray ionization, peptide masses were analyzed in an Orbitrap Fusion mass spectrometer (Thermo Scientific, San Jose, CA, USA) operated in data-dependent mode. Further details of the applied method can be found in the supplementary materials section.

LC-MS/MS data were analyzed using Proteome Discoverer (v1.4.1.14, Thermo Scientific). MS/MS spectra were searched against the *S. multivorans* database containing 3,191 non-redundant protein-coding sequence entries (NCBI Genbank, accession number CP007201.1) using the SEQUEST HT and MS Amanda search engines with the following settings: trypsin as cleaving enzyme, oxidation of methionine as dynamic and carbamidomethylation of cysteine as static modification, up to two missed cleavages, MS mass tolerance set to 10 ppm and MS/MS mass tolerance to 0.05 Da. False discovery rate for peptides was set to <0.01 (Supplement information Table S1). Quantification of proteins was performed using the average of top 3 peptide area (Bondarenko *et al.*, 2002) as implemented in ProteomDiscoverer 1.4. Protein quantification was considered successful for proteins recovered in >50% of biological replicates, otherwise they were classified as identified but not quantified proteins. After log10 transformation, the protein abundance values were normalized to the median and bioinformatic analysis was applied by principal component analysis and T-test statistics (R). Significance threshold $p<0.05$ in a two-tailed test were considered as significantly altered.

Tetrachloroethene reductive dehalogenase (PceA) activity measurement

The main cultures were harvested in the exponential growth phase by centrifugation (12,000 x g, 10 min at 10°C). The cells were resuspended in 2 volumes of anaerobic buffer (100 mM Tris-HCl pH 7.5) and disrupted using a French Press (6.9 MPa = 1000 psi). Defined amounts of the cell extracts were transferred to anoxic vials (nitrogen atmosphere) closed with rubber stoppers. In the vials different oxygen concentrations (0%, 1%, 2.5%, 5% and 20%) were adjusted by transferring defined amounts of air into the gas phase with a syringe. The oxygen concentrations in the gas phase were monitored with a Microx4 oxygen meter (PreSense-Precision Sensing GmbH, Regensburg, Germany). The reaction batches were incubated at 28°C under stirring. At defined time points the activity of PceA was measured as described earlier using a photometric assay with reduced methyl viologen as artificial electron donor (Neumann *et al.*, 1996).

PCE dechlorination in cell suspensions (resting cells)

As reaction medium the defined mineral medium (see above) with pyruvate (40 mM) but without yeast extract, resazurin, cysteine, iron and trace element solution was used. The experiment was conducted in 200 mL rubber-stoppered glass serum bottles. To adjust specific oxygen concentrations in the reaction medium (0%, 2.5%, 5% and 7.5% oxygen) air was injected with a syringe to 100 ml medium in a 200 mL serum bottle. To dissolve the oxygen completely in the medium, the bottle was shaken rigorously (300 rpm) for 6 hours. The oxygen-enriched medium of two bottles was then combined for the experiment to ensure a gas phase as small as possible.

PCE (200 µM) was added to the medium and the reaction batch was stirred overnight and subsequently incubated for one hour at 28°C. The reaction batches were inoculated with 5% of a *S. multivorans* culture or a *D. hafniense* Y51 culture. The cultures used for inoculation were in the mid-exponential growth phase. The reaction batches were incubated at 28°C while stirring. Samples were taken every 20 minutes.

Purification of PceA

The PceA enzyme was purified as described before (Bommer *et al.*, 2014) from a *S. multivorans* mutant strain producing strep-tagged PceA (PceA-Strep). *S. multivorans* was cultivated with Pyruvate/PCE as described above, but with kanamycin (100 µg/ml) and an elevated iron content (720 µM). The ratio of aqueous to gas phase was 1:1. The cultures were harvested in the exponential growth phase by centrifugation (12,000 x g, 10 min at 10°C). All steps of the enzyme purification where conducted under anoxic conditions. The cells were disrupted using a French Press (6.9 MPa = 1000 psi). PceA-Strep was purified from cell extracts via gravity flow using the Strep-Tactin Superflow column material (IBA, Göttingen, Germany). The Strep-Tactin column was pre-equilibrated with buffer A (100 mM Tris-HCl pH 8.0), which was also used for washing steps. PceA-Strep was eluted from the column material with buffer B (100 mM Tris-HCl pH 8.0, 2.5 mM desthiobiotin). The purified PceA was concentrated using a Vivaspin 6 (30 KDa) ultrafiltration unit (Sartorius, Göttingen, Germany) and then transferred into small anoxic rubber-stoppered glass vials (0% oxygen condition) or open Eppendorf tubes (20% oxygen condition). The reaction batches were incubated at 28°C. At defined time points the PceA activity was determined as described above.

Analysis of metabolites

Dechlorination of PCE was monitored by gas phase GC-FID analysis of culture samples using a Clarus 500 gas chromatograph as described in (Mac Nelly *et al.*, 2014) The oxygen concentration in the medium and in the gas phase during oxygen respiration in large bottles was measured via fluorescence intensity changes of oxygen sensitive optode spots (POF-PfSt3, Presens, Regensburg, Germany) and a corresponding detection device from the same company (Fibox 3 LCD trace v7).

In other experiments, the oxygen concentration was measured with the Microx4 oxygen meter and a needle-type oxygen microsensor NTH-PSt7 (PreSense-Precision Sensing GmbH, Regensburg, Germany). Temperature measurement was carried out in separate replicates. The oxygen sensor had a measurement range of 0% - 100% (0 – 45 mg/l) oxygen and a detection limit of 0.03% oxygen.

Redox measurements and redox potential adjustment

The redox potential of the medium in growing cultures was adjusted using a 3-electrode-potentiostat (Wenking LB 81 M, Bank Elektronik, Göttingen, Germany) with a graphite working electrode. The redox potential was adjusted against an $Ag^+/AgCl$ reference electrode (Ref100, Unisense A/S, Århus, Denmark) with a standard potential of +207 mV. Redox potentials in the medium were measured with a redox electrode (Pt4800-M5-S7, Mettler Toledo, Gießen, Germany) on a pH meter. Before inoculation, the medium was made anoxic and adjusted to the defined redox potential. The experiments were performed under anoxic conditions.

Results

PCE is dechlorinated by *S. multivorans* under microoxic conditions

In a previous study, *S. multivorans* was shown to grow with 5% oxygen as electron acceptor (Goris *et al.*, 2014). Here, we measured the redox potential of the medium with 5% oxygen to be approximately +230 mV (E°' vs. the hydrogen electrode; data not shown). Since it was not known whether oxygen or the redox potential or both affect the organism's ability to respire PCE, the influence of the redox potential on PCE respiration was tested in the absence of oxygen. Therefore, stable redox potentials in the range of -600 to +400 mV were set with a potentiostat in anaerobically PCE-grown cultures. The redox potential had no significant influence on growth or PCE dechlorination as detected by *c*DCE formation (Supplemental Figure S1). To assess if PCE can be dehalogenated under oxic conditions, *S. multivorans* was grown with O_2 concentrations of 5%, 2% and 1% in the gas phase corresponding to measured aqueous concentrations of 1.86 mg/l, 0.746 mg/ml and 0.373 mg/ml, respectively. The experiment was performed in rubber-stoppered 2 L glass bottles with a gas to aqueous phase ratio of 9:1. The bottles were shaken to ensure optimal oxygen transfer from gas to liquid phase. The medium was amended with PCE dissolved in hexadecane (4 ml per 200 ml culture; 10 mM nominal PCE concentration corresponding to about 200 µM dissolved PCE in the medium). The oxygen concentration in the aqueous phase was measured during growth. PCE dechlorination was not detected at oxygen concentrations of 5% or 2%. With 1% initial oxygen in the gas phase, the cells started to dechlorinate PCE at significant rates as soon as the oxygen concentration dropped to less than about 0.5% (Supplemental Figure S2).

Since the used optode spots did not allow for accurate measurement of oxygen concentrations in the medium below 0.2 mg/ml, in a second approach cultures were grown in a hypoxic chamber, where oxygen concentrations in the atmosphere were set to a constant value of 0.2, 0.5 or 1%. To ensure optimal equilibration of the gas phase with the medium, *S. multivorans* was grown under shaking in Erlenmeyer flasks with aluminum caps (no septa) and a high gas phase to medium ratio of 9:1. The formation of the PCE dechlorination products trichloroethene (TCE) and cDCE was observed up to an oxygen concentration of 0.5% but not at 1% (Table 1). Product formation started approximately one hour after starting the experiment. The quantification of PCE dechlorination was not possible, since a major portion of the chlorinated ethenes evaporated from the liquid phase under the experimental conditions applied. The minimal detection limit of cDCE and TCE was 0.5 µM. All four replicates showed formation of cDCE and TCE at an oxygen concentration of 0.2%, while TCE was the only dechlorination product detected in two of the four

samples at an oxygen concentration of 0.5% (Table 1). From all these observations it can be concluded that *S. multivorans* is able to dechlorinate PCE at oxygen concentrations of up to approximately 0.5% in the gas phase, corresponding to 0.19 mg/ml dissolved oxygen in the liquid phase. *Desulfitobacterium hafniense* Y51, a PCE-respiring obligate anaerobe from a genus known to tolerate low amounts of oxygen (Madsen & Licht, 1992, Utkin *et al.*, 1994, Kim *et al.*, 2012), did not dechlorinate PCE in the presence of any of the tested oxygen concentrations in both experimental set-ups (see Table 1).

PCE dechlorination in cell suspensions of *S. multivorans* in the presence of O_2

To investigate PCE dechlorination under oxic conditions in cell suspensions (resting cells), *S. multivorans* cells were transferred into a pyruvate/PCE/oxygen-containing solution similar to the growth medium, but lacking essential supplements to exclude growth. Cell suspensions without oxygen were used as positive control. Initial oxygen concentrations of 2.5% (corresponding to 0.932 mg/l in the medium), 5% (1.864 mg/l) or 7.5% (2.796 mg/l) in the gas phase were applied. The dechlorination of PCE started in cell suspensions (approximately 20 µg/ml cell protein) without oxygen directly after the inoculation of the reaction mixture with *S. multivorans* (Figure 1A). In contrast, in cell suspensions with oxygen the dechlorination of PCE did not start before oxygen was depleted to concentrations below 0.19 mg/l (0.5%) (Figure 1B). The oxygen concentration in the medium decreased to a constant level of approximately 0.1mg/ml as measured with a needle-type oxygen microsensor (see materials and methods). The oxygen concentration in the control with anoxic medium was below the detection limit (0.01mg/ml).

The cell suspension experiments were also performed with *D. hafniense* Y51 cells. With initial oxygen concentrations of 5% and 7.5%, neither the oxygen nor the PCE concentration decreased. When using lower oxygen concentrations of 1% or 2.5%, a decrease of oxygen to <0.03% or 0.7%, respectively, was measured. PCE dechlorination was only observed in the experiment with an initial oxygen concentration of 1% after oxygen was depleted (Supplemental Figure S3).

Oxygen sensitivity of the reductive dehalogenase PceA

Since PCE dechlorination by *S. multivorans* was observed at low oxygen concentrations, we tested to what extent the PCE dehalogenase PceA, the key enzyme of PCE dechlorination, is oxygen-stable. This was of special interest, since in general reductive dehalogenases, including PceA, are described as oxygen-sensitive (Neumann *et al.*, 1996, Holliger *et al.*, 1998). Therefore, we measured PCE dechlorination photometrically in crude extracts that had been exposed to different concentrations of oxygen (0%, 1%, 2.5%, 5% and 20% in the gas phase; 1% corresponds to measured 0.37 mg/ml in the medium) prior to the activity test. PceA activities were measured at defined time points after starting the experiment using methyl viologen as artificial electron donor (redox potential E°' = -446 mV, Figure 2A). The initial PceA activity for all preparations was approximately 80 nkat mg^{-1}. With 0% or 1% oxygen in the gas phase, the PCE dechlorination remained constant for about 96 hours (Figure 2A). At higher oxygen concentrations, the enzyme activity decreased rapidly at a rate that was dependent on the oxygen concentration (e.g. 2.5 hours with 5%/1.9 mg/ml oxygen).

The same experiment was repeated with cell extracts of *D. hafniense* Y51. Even in the absence of oxygen, the enzyme was not stable, losing 50% of its dehalogenation activity after 6 hours, while after 24 hours only about 30% of the initial PceA activity remained (Figure 2B). In the preparations containing 1% and 2.5% oxygen (0.37 and 0.93 mg/ml, respectively), the enzymatic half-life was 3 hours. Taken together, PceA of *S. multivorans* is more stable under low oxygen concentrations (1%) than the enzyme of *D. hafniense* Y51. Oxygen concentrations of 5% (1.86 mg/ml) or more lead to rapid inactivation of the enzyme of both organisms.

Purified PceA of *S. multivorans* showed a half-life of about 2 hours when exposed to atmospheric concentrations of oxygen (Supplemental Figure S4), which is similar to the values given in the literature (Neumann *et al.*, 1996).

Proteome response to oxygen exposure

To examine the proteomic response of *S. multivorans* to oxygen, label-free quantitative proteomics with cells grown under different conditions was performed. The aim of the proteome analysis was to analyze 1) the global proteome of *S. multivorans* respiring oxygen, 2) the global proteome (especially stress) response to atmospheric oxygen concentrations and 3) specifically, the influence of oxygen on the induction of proteins involved in PCE respiration. *S. multivorans* cells grown anoxically with PCE as electron acceptor were used as standard condition.

In total, 1,633 distinct proteins were identified under at least one of the applied cultivation conditions (supplemental Table S1, data sheet S1), corresponding to 51% coverage of the annotated 3,191 non-redundant protein-coding sequences of *S. multivorans*. Among all conditions, 1,888 proteins could be identified of which 1,640 were quantified. Between 1,084 and 1,451 proteins were quantified in *S. multivorans* cells under the different experimental setups.
A principal component analysis (PCA) of quantified proteins was performed as indicator of the variance between experimental conditions and replicates in the dataset. We observed a distinct separation of the datasets for cultures grown in the presence and absence of oxygen and with different oxygen concentrations (Supplemental Figures S5A and B). When investigating the induction of organohalide respiration in the presence of oxygen in experiment 3 (see Figure 3), samples of cells harvested early (O_2 early) show a wider distribution of the replicates in the PCA compared to the samples harvested at the second time point (Supplemental Figure S5C). This can most likely be attributed to the heterogeneity of the cultures in the early exponential growth phase.

Experiment 1 + 2: Response to oxygen respiration and to atmospheric oxygen concentration

In the first experiment, the proteomes of *S. multivorans* cells grown by PCE respiration or O_2 respiration were analyzed and compared to each other after harvesting the cells in the mid-exponential phase. The protein abundance value (mean of top 3 peptide area) of 35 quantified proteins differed significantly between the two different growth conditions (Supplemental Table S1, data sheet S1).The proteins encoded by genes in the organohalide respiration region were found to be present exclusively with PCE, as expected and found previously also in comparison to cells grown with fumarate or nitrate as electron acceptors (Goris *et al.*, 2015).
The main enzyme complexes involved in the oxygen respiration, cytochrome cbb3 oxidase (encoded by SMUL_2652-2655) and cytochrome c reductase (bc1 complex, encoded by SMUL_2521-2525) were found in similar amounts in the two compared proteomes. (see Table 2 and Supplemental Table S1, sheet S2) in oxygen-grown cells when compared to PCE-grown cells. The two distinct complexes I found in *S. multivorans*, 1) the NADH menaquinone oxidoreductase and 2) an epsilonproteobacterial type, a putative ferredoxin menaquinone oxidoreductase, were also found in similar amounts (Table 2). An uncharacterized cytochrome c-like membrane protein (encoded by SMUL_750) with 445 amino acid residues and 8 transmembrane helices showed

higher abundance in oxygen-grown cells than in PCE-grown cells (6-fold higher, p-value 0.002), but it is not known whether it participates in a respiratory chain or has a different function.

Two proteins putatively involved in oxygen-related stress response were found to be more present with oxygen: a cytochrome c551 peroxidase belongs to one of the most differentially quantified proteins (SMUL_0575, 16-fold), while a putative superoxide reductase was found at four-fold increased levels (SMUL_1334) in oxygen-containing cultures (Table 2). The heat shock protein Hsp20 (SMUL_0547), which is thought to be involved in stress response to PCE in *S. multivorans* (Goris *et al.*, 2015), was down-regulated (6.4 times) in cells using oxygen as electron acceptor instead of PCE.

Several changes were also found in proteins involved in the TCA cycle. A class II fumarate hydratase (SMUL_1459) was found in a 3.4-times higher amount in oxygen-grown cells compared to cells grown with PCE as electron acceptor, while the Fe-S cluster-containing class I fumarate hydratase (SMUL_1679/1680) was not found in significantly different amounts. This is reasonable, as the class II enzymes are known to function under oxic conditions (Tseng *et al.*, 2001). Additionally, the two malate dehydrogenases (MDH) encoded in the *S. multivorans* genome were found to be more abundant in the proteome of oxygen-grown cells. The MDH encoded by SMUL_0065 was only found in oxygen-grown cells, while the isoenzyme (SMUL_1443) was present in higher amounts (~2.6-fold, p-value 0.008) as compared to PCE-grown cells. The malate-quinone oxidoreductase amount was not significantly altered. Pyruvate is channelled into the TCA cycle via acetyl-CoA by pyruvate ferredoxin oxidoreductase. This enzyme was not found in altered amounts, but an alternative, pyruvate-oxidizing enzyme, the quinone-dependent pyruvate dehydrogenase, was found 8-fold increased (p-value 0.005) in oxygen- than PCE-grown cells.

Two proteins highly up-regulated in the presence of oxygen are not connected directly to pyruvate-dependent oxygen respiration. With a more than 20-fold higher abundance in oxygen-grown cells (p-value 0.04), the catalytic subunit of a molybdopterin oxidoreductase (SMUL_950) was the protein with the highest increase. A small subunit (SMUL_951) was found only in oxygen-grown cells. The molybdopterin oxidoreductase belongs to the family of MopB3 proteins, which is uncharacterized. A blast search revealed that similar enzymes are encoded in eight of the eleven sequenced *Sulfurospirillum* spp. genomes (except *S. arcachonense*, *S. barnesii* and *S. deleyianum*) as well as in Deltaproteobacteria and Enterobacteria. A TAT signal was predicted, suggesting the protein to be periplasmic.

Lactate utilization protein subunits ABC (SMUL_1033-1035), shown to be responsible for oxygen-dependent lactate oxidation in *Campylobacter jejuni* and *Shewanella oneidensis* (Pinchuk *et al.*,

2009, Thomas *et al.*, 2011), were found among the proteins with the highest upregulation (3.7 to 7.6-times).

Experiment 2 was designed to test the influence of atmospheric oxygen concentrations, under which *S. multivorans* is not able to grow (Goris *et al.*, 2014). Cultures were first grown anoxically with PCE as electron acceptor until mid-exponential growth phase and then exposed to either 20 or 5% oxygen in the gas phase Growth of cultures exposed to 5% oxygen was similar to control cultures (pyruvate/PCE without oxygen, data not shown) while the culture exposed to 20% oxygen stopped growing (Figure S6). Cells were harvested after being exposed 6.5 hours to oxygen. Eighteen proteins were significantly regulated when comparing both conditions. Four of these were found to be higher abundant when cells were exposed to 20% oxygen (Table 2, Supplemental Table S1, data sheet S4). Of these four proteins, one is predicted to be functionally related to oxygen stress response, an alkyl hydroperoxide reductase AhpC (SMUL_3225), with 6.2-times higher amounts than in cells exposed to 5% oxygen. A catalase, not found in any other proteome investigated in this study, was detected at low levels (abundance of 6.8, in the lowest quartile of quantified proteins) in cells incubated with 20% oxygen.

The two superoxide dismutases encoded by *S. multivorans* SMUL_0529 (Fe-type) and SMUL_3084 (Cu-Zn-type) were not found in significantly different amounts. The first one is found in the top 25% of proteins from cells grown with 5% oxygen (value of 8.5), the latter in the bottom 30% (7.0)

Surprisingly, several proteins belonging to the down-regulated proteins of cells exposed to 20% oxygen were found to be involved in oxygen respiration. Among these are the *bc1* complex and the cytochrome *c* oxidase. Additionally, the lactate utilization proteins and the unknown cytochrome c, which were found to be up-regulated with 5% oxygen when compared to PCE-grown cells, were found to be down-regulated with 20% oxygen. This might be due to a shutdown of the oxygen-respiratory chain to prevent damage from radical oxygen species.

Experiment 3: Induction of PCE respiration in the presence of oxygen

The influence of oxygen exposure on induction of PCE respiration in OHR-down-regulated cells was investigated by inoculating OHR-down-regulated cells (grown without PCE for more than 60 transfers) in medium containing 10 mM PCE only or PCE plus oxygen (5%) as electron acceptor. The latter cultures reached the stationary growth phase within 88 hours and reached an optical density of about 0.47. At this time point, three of six cultures fed with oxygen and PCE were harvested for proteomic and biochemical studies (designated O_2 early), the other three cultures

were further incubated and harvested later (O_2 late) (Figure 3B). PCE dechlorination started 135 hours after inoculation in both cultures (PCE/5% oxygen and PCE only) (Figure 3A/B). After another 48 hours, when PCE was completely depleted in the PCE only culture (= exponential growth phase) (Supplemental Figure 7A) and 72% of the PCE was depleted in the O_2 late culture (Supplemental Figure 7B), both cultures were harvested. In all samples, PceA was monitored by Western Blot analysis as well as by measuring enzymatic PCE dechlorination activity. Active reductive dehalogenase PceA was detected in all cultures harvested after 183 hours of cultivation, although at a much lower level in the PCE/oxygen-grown cells as shown in a Western blot of the respective cultures (Supplemental Figure S8).

In cultures harvested directly after oxygen consumption (O_2 early), only PceA, the two-component response regulator (SMUL_1539) and a putative FMN-binding protein (SMUL_1576) of the components in the region encoding proteins for organohalide respiration (OHR region) could be quantified (Figure 4, Supplemental Table S1, data sheet S8). While the response regulator (SMUL_1539) was quantified in similar amounts as in cells grown with PCE as electron acceptor, the reductive dehalogenase PceA as well as the FMN-binding protein were detected in more than 100 times lower amounts. In the O_2 late cells, nine proteins of the OHR region (PceA, the regulator, an IscU-like protein and seven proteins inside or downstream the corrinoid biosynthesis gene cluster) could be quantified in contrast to 28 proteins encoded by this region in cells grown with PCE only (6, Supplemental Table S1, data sheet S8). Additionally, seven corrinoid biosynthesis proteins were identified in one replicate. Seven of the nine quantified OHR proteins were up-regulated (5 to 16 times) in cells grown with PCE only compared to the O_2 late cells (Figure 4, Supplemental Table S1, data sheet S6).

Discussion

Reductive dechlorination of PCE in the presence of oxygen

The observation of *S. multivorans* being one of the very few microaerophilic OHRB (Goris *et al.*, 2014), led to the speculation that the organism might be able to reductively dehalogenate chlorinated or brominated alkenes in the presence of oxygen. The fact that reductive dehalogenation was completely independent of the environmental redox potential up to +400 mV came as a surprise, as in general the redox potential suitable for supporting organohalide respiration was thought to be lower. Whether reductive dehalogenation is mediated under high redox potentials due to *S. multivorans* actively lowering the periplasmic potential or whether reductive dehalogenation itself is independent of the redox potential cannot be concluded here.

Although the quantification of reductive dechlorination of PCE in the presence of low amounts of oxygen was difficult to achieve, we gathered results of three different experiments, which all point toward the reductive dehalogenation being mediated at up to about 0.19 mg/ml oxygen (0.5% in the gas phase). This could be of importance in the oxic-anoxic phase of PCE-contaminated environments with oxygen concentrations close to zero. Above 0.5% oxygen, PCE dechlorination was inhibited. The reason for this is most probably not because of fast down-regulation of the PCE respiration proteins, since the down-regulation of PceA in the presence of an alternative electron acceptor and in the absence of PCE requires a long time also when oxygen is used as electron acceptor (John *et al.*, 2009, unpublished data). Since the key enzyme of the reductive dehalogenation, the corrinoid and Fe-S cluster-containing PceA, exhibited oxygen tolerance for at least 48 hours with 1% oxygen in cell extracts, it might be not the only limiting factor in PCE dechlorination in the presence of oxygen. This is in contrast to PceA of *D. hafniense* Y51, which exhibited higher oxygen sensitivity at an oxygen concentration of 1%. Probably due to this, *D. hafniense* Y51 did not show any PCE dechlorination in the presence of oxygen.

Since the PCE respiratory chains of both organisms likely involve other redox-active proteins, it is feasible that these are even more oxygen-sensitive. This might apply to the PCE-induced putative quinol dehydrogenase of *S. multivorans* (Goris *et al.*, 2015), as the amino acid sequence of its periplasmic subunit contains four [4Fe-4S] cluster binding motifs (Goris *et al.*, 2014).

Key enzymes for oxygen respiration

The key enzymes of oxygen respiration, namely a cbb3-type cytochrome *c* oxidase and an ubiquinol-chytochrome *c* reductase, were found in all cultures independent of the electron

acceptor, pointing toward a constitutive expression of these complexes. However, in the PCE induction experiment, where the O_2 early cultures were harvested directly after depletion of oxygen and the O_2 late cultures were harvested when the oxygen was already consumed for a longer period of time, the cytochrome c oxidase was found in higher amounts. This could be due to a possible up-regulation of this high-affinity oxidase in environments with very low or changing amounts of oxygen as found for other bacteria (Loisel-Meyer *et al.*, 2005, Colburn-Clifford & Allen, 2010).

S. multivorans incubated with 20% oxygen stopped growing and enzymes functionally related to oxygen stress response could be detected in the proteome. Considering this physiological reaction as well as at the proteome analysis of *S. multivorans*, a shutdown of catabolism due to oxidative stress can be assumed, as this would generate lesser reactive oxygen species (ROS).

Enzymes mediating resistance to oxidative stress

In an oxic atmosphere, bacteria are confronted with toxic metabolic byproducts, since a portion of oxygen molecules are converted to ROS (Ezraty *et al.*, 2017). Typical compounds causing oxidative stress are oxygen, superoxide anions, hydroxyl radicals and hydrogen peroxide. To resist oxidative stress, bacteria have evolved a variety of scavenging enzyme systems including superoxide dismutase (SOD), superoxide reductases (SOR), catalase and cytochrome *c* peroxidase (Ccp) (Ezraty *et al.*, 2017), which are all encoded in the genome of *S. multivorans*. Two of these enzymes seem to be up-regulated with oxygen as electron acceptor in *S. multivorans*, a cytochrome *c*551 peroxidase and a putative superoxide reductase. Superoxide reductases (SOR) reduce superoxide to hydrogen peroxide (Sheng *et al.*, 2014), while cytochrome *c* peroxidases are assumed to catalyze the conversion of hydrogen peroxide to water when cells are stressed with oxygen (Atack & Kelly, 2007). Therefore, the upregulation of both enzymes at the same time makes sense, especially since catalase, the enzyme usually employed to detoxify hydrogen peroxide, was not found in cells grown with 5% oxygen. The only condition under which catalase was detected was a culture incubated with 20% oxygen. This is in line with observations in *E. coli*, where catalase is recruited when hydrogen peroxide levels are high (Imlay, 2013). The two superoxide dismutases (Fe-type and Cu-Zn-type), which convert superoxide anions to hydrogen peroxide and oxygen, were found in similar amounts in PCE-grown and oxygen-grown cultures. The Fe-type SOD was shown to be present in higher amounts with PCE as electron acceptor when compared to nitrate or fumarate in a previous study (Goris *et al.*, 2015). Therefore,

it is feasible that the Fe-SOD is part of a general stress response induced with PCE or oxygen in *S. multivorans*. The Cu-Zn-type enzyme seems to be of minor importance under the tested conditions when compared to the Fe-type SOD, since the latter was found in much higher amounts.

AhpC, found in higher amounts in cultures incubated with 20% oxygen, is the peroxide-reducing protein of the alkyl hydroperoxide reductase system and a representative of the enzyme class of peroxiredoxins (Prx) characterized in Enterobacteria and Mycobacteria (La Carbona *et al.*, 2007). These enzymes act in response to hydrogen peroxide stress by converting hydrogen peroxide and organic hydroperoxides to water and the corresponding alcohols, respectively (Wood *et al.*, 2003). AhpC was shown to reduce organic hydroperoxides in the Epsilonproteobacterium *Wolinella succinogenes* (Kern *et al.*, 2011). In the same study it was also stated to be unlikely that AhpC contributes significantly to the stress response induced directly by hydrogen peroxide. In *S. multivorans* it could have a similar role in getting rid of organic hydroperoxides which might be formed at high oxygen levels. AhpC is thought to be dependent on its reduction by one of the several cell-specific disulfide oxidoreductases-dependent redox proteins such as thioredoxin (Trx), AhpF or AhpD. In Epsilonproteobacteria, including *W. succinogenes*, TrxA is the most likely electron donor for the different peroxiredoxins (Atack & Kelly, 2009, Kern *et al.*, 2011). An orthologue to TrxA of *W. succinogenes* is encoded in the genome of *S. multivorans* (SMUL_232) and highly expressed under all conditions, although not significantly higher abundant with oxygen. Nonetheless, it might function to reduce AhpC in *S. multivorans*.

The significant higher amount of a putative periplasmic molybdopterin oxidoreductase in oxygen-grown cells is puzzling, since many enzymes of this group are involved in anaerobic energy metabolism (Grimaldi *et al.*, 2013). While the molybdopterin enzyme xanthine dehydrogenase is discussed to take part in stress response of animals (Schwarz *et al.*, 2009), a similar role in bacteria has not been found so far. However, since molybdopterin oxidoreductases are often involved in transferring oxygen atoms (mainly acquired from water) (Pushie *et al.*, 2014), a function in the reduction or oxidation of potentially harmful oxidized inorganic or organic molecules in the periplasm is not unlikely. Similarly unexpected was the upregulation of lactate utilization proteins ABC (SMUL_1033 – 1035) in oxygen grown cells of *S. multivorans*, since lactate was not added to the medium. The proteins show sequence identities (32% of protein B and 43 % identity to protein C) to the characterized L-lactate dehydrogenase (encoded by NMB_1436-1438) of *Neisseria meningitidis* (Grifantini *et al.*, 2004). The lactate dehydrogenase of *N. meningitides* has also been shown to be responsible for the protection of cells against hydrogen peroxide-mediated

killing. The lactate utilization proteins of *S. multivorans* might have a similar function in the defense of hydrogen peroxide-mediated stress.

Conclusion: With the current study we showed for the first time, that *Sulfurospirillum multivorans* can dechlorinate PCE in the presence of oxygen concentrations below 0.5%. In contrast to the anaerobic bacterium *Desulfitobacterium hafniense* Y51, the facultative microaerophilic PCE-respiring *Sulfurospirillum multivorans* has a more oxygen-stable PCE reductive dehalogenase, functioning *in vivo* and *in vitro* under micro-oxic conditions. The proteomic response of *S. multivorans* to oxygen revealed two main enzymes which are up-regulated as a stress response when the organism respires oxygen, a superoxide reductase and a hydrogen peroxide reductase. The findings of this study are important in studies on reductive dehalogenation in oxic-anoxic zones and gives insight into the proteomic response of microaerophilic Epsilonproteobacteria to oxygen.

Funding: This work was supported by the German Research Foundation (DFG), as part of the research unit FOR 1530. The authors used the analytical facilities of the Centre for Chemical Microscopy (ProVIS) at the Helmholtz Centre for Environmental Research, which is supported by European Regional Development Funds (EFRE - Europe funds Saxony) and the Helmholtz Association.

Acknowledgement: Benjamin Scheer is thankfully acknowledged for technical assistance and maintenance of the Orbitrap Fusion mass spectrometer.

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Main Figures and Tables

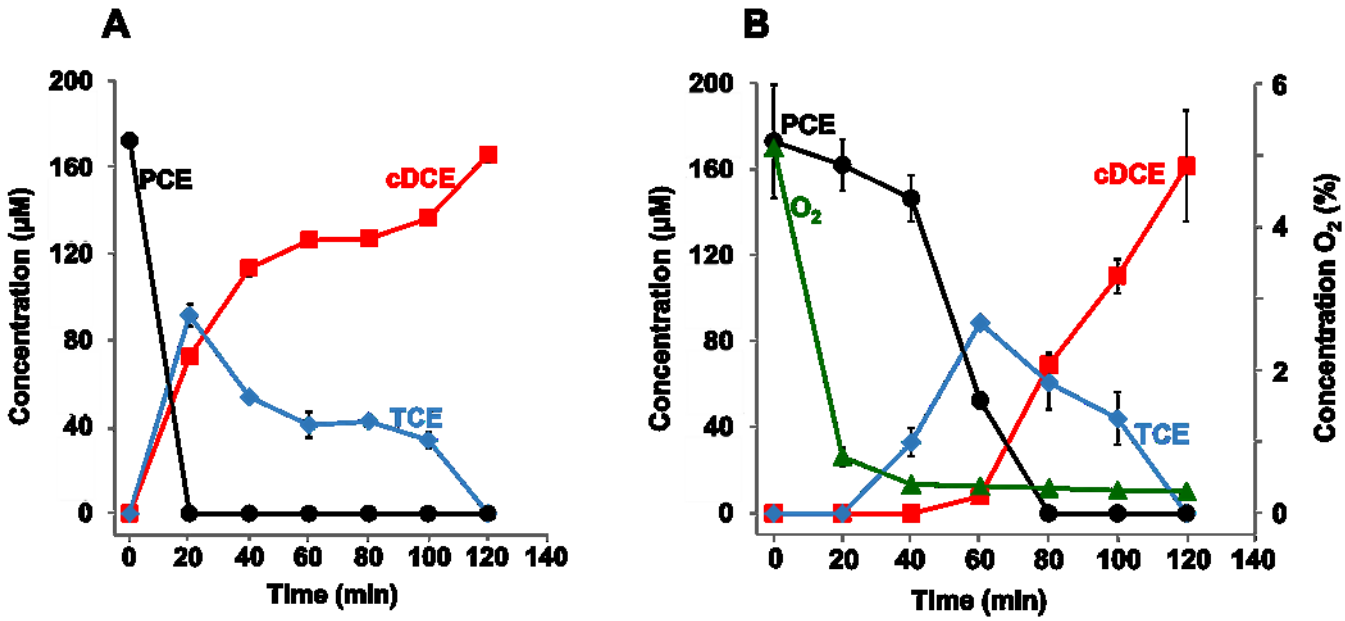

Figure 1: **Dechlorination of PCE by cell suspensions of *S. multivorans* in the presence of different oxygen concentrations**. **A**: Dechlorination of PCE and formation of cDCE and TCE in the absence of oxygen. **B**: Dechlorination of PCE and formation of cDCE and TCE as well as the consumption of oxygen. The initial oxygen concentration was 5% in the gas phase (as measured to be 1.86 mg/l in the medium); the protein concentration was 12 – 20 µg/ml. Measurements were carried out in biological triplicates.

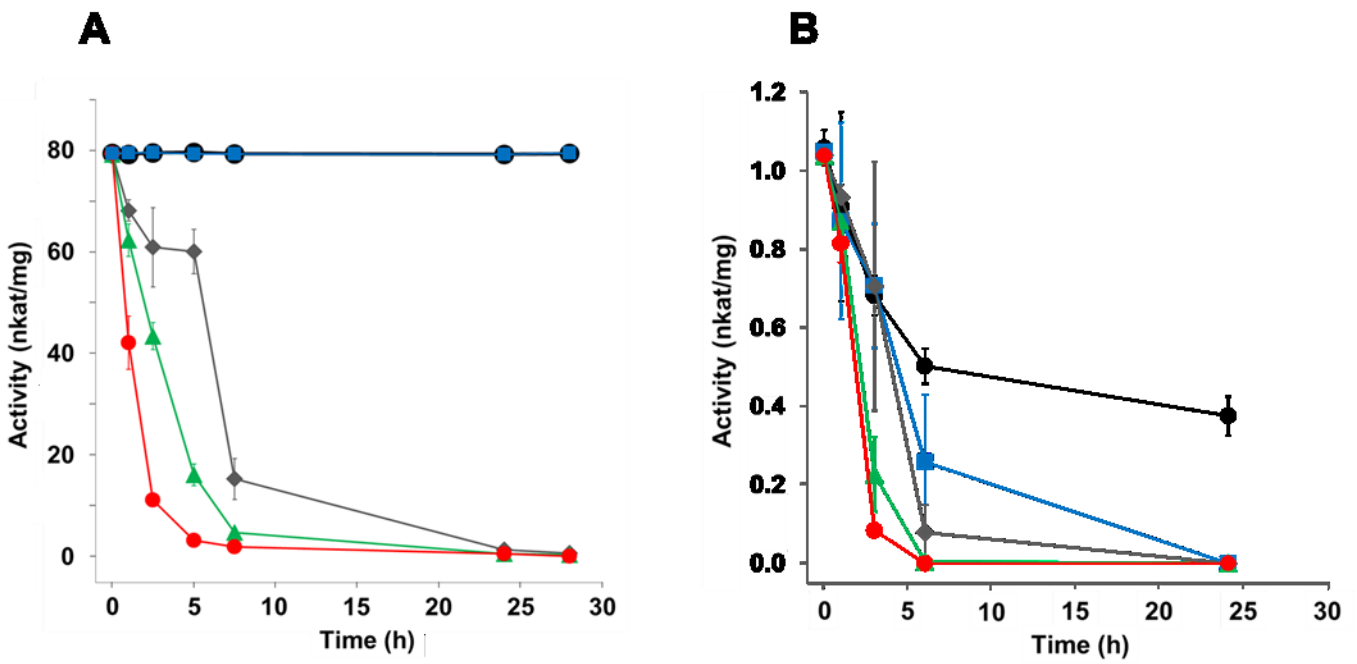

Figure 2: Activity of the reductive dehalogenase PceA of *S. multivorans* and *D. hafniense* Y51 in the presence of different oxygen concentrations applied to crude extracts before measurement. **A**: PceA activity of crude extracts of *S. multivorans*. **B**: PceA activity of crude extracts of *D. hafniense* Y51. The crude extracts were incubated at 28°C under stirring and were exposed to the oxygen concentrations indicated. Oxygen concentrations: black curve = 0% O_2, blue curve = 1% O_2 (measured as 0.37 mg/ml in the medium), grey curve = 2.5% O_2 (0.93 mg/ml), green curve 5% O_2 (1.86 mg/ml) and red curve = 20% O_2 (7.44 mg/ml). Measurements were carried out in biological as well as technical triplicates.

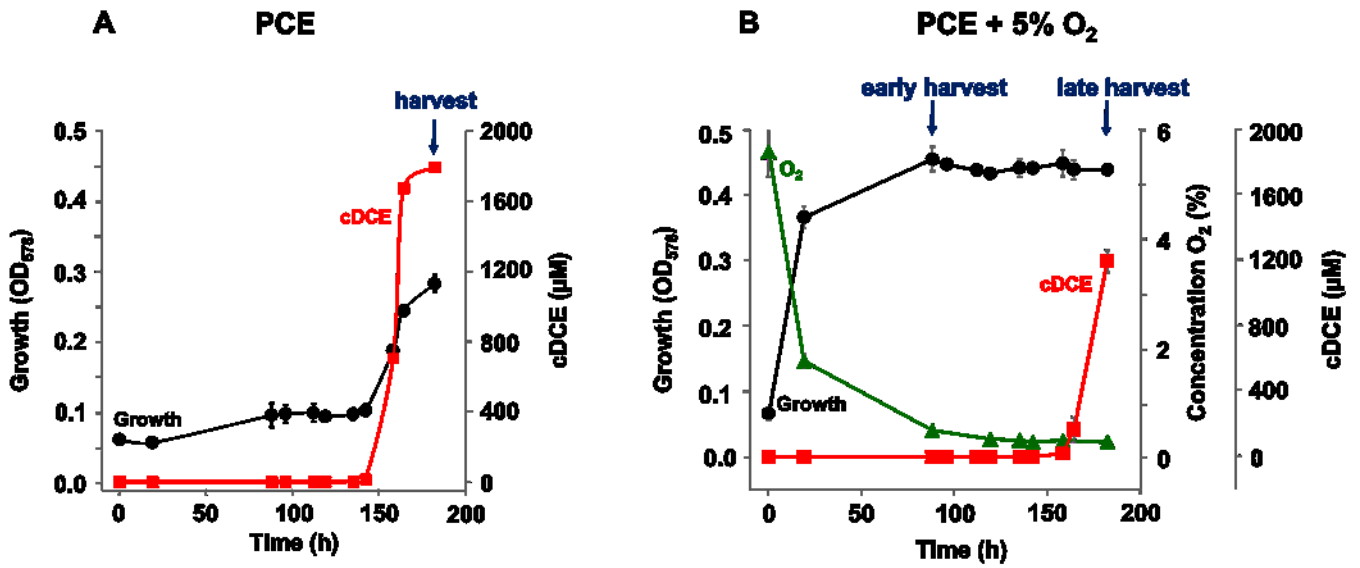

Figure 3: Induction of PCE respiration in the presence of oxygen. For the induction of organohalide respiration in *S. multivorans* in the presence of oxygen, cells with down-regulated PCE respiration were used (see Materials and Methods). Pyruvate served as electron donor for all cultures. **A**: As control cells three cultures were grown with PCE as sole electron donor. **B**: Six cultures were grown with PCE + 5% O_2. Three of these cultures were harvested in the early stationary growth phase (early) while the other three cultures were harvested in the late stationary growth phase (late) when the cells had started PCE dechlorination. The time point at which PCE dechlorination started is indicated by a red arrow, the time points at which cells were harvested are indicated by dark-blue arrows.

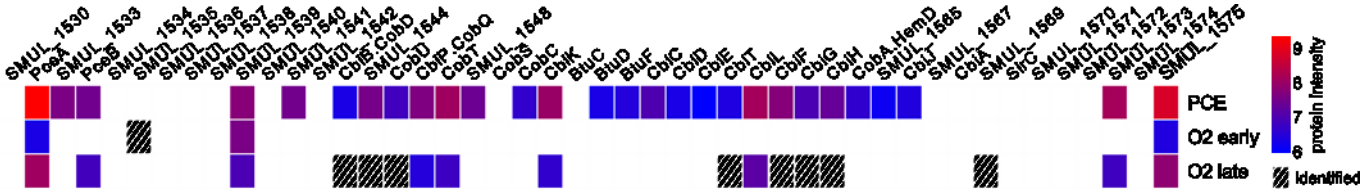

Figure 4: Identified gene products of the OHR core region as detected by proteomic analysis. Each square correlates to a given gene product identified or quantified under given cultivation/experimental condition (at the right). For quantified proteins the protein intensity is provided (color code at the far right, normalized and logarithmized average of top 3 peptide area as described in the methods section), proteins identified but not quantified are represented by shaded squares.

Table 1: Dechlorination of PCE in cultures of *S. multivorans* with different constant oxygen concentrations. The formation of TCE and cDCE was measured by GC. The experiment was performed in a hypoxic chamber. +: formation of cDCE and/or TCE (only values of at least 50 nmol were considered), respectively; -: no formation of dechlorination products detected.

Oxygen concentration	*S. multivorans*		*D. hafniense* Y51	
	Formation of			
	TCE	cDCE	TCE	cDCE
0.2%	+	+	-	-
0.5%	+	-	-	-
1.0%	-	-	-	-

Table 2: Level changes of proteins involved in oxygen respiration, moxic stress response (experiment 1), or stress response to atmospheric oxygen concentration (experiment 2). Proteins marked in orange are significantly up- or down-regulated (fold change of +/- 2.82, p-value ≤ 0.05).

		1	2
		5% O_2 vs. PCE	20% O_2 vs. 5% O_2
Locus Tag SMUL_	Description	Fold Change	Fold Change
0195-0203	NADH ubiquinone oxidoreductase	-1.92 - 1.40	-2.58 - -1.07
0333	cold shock protein CspA	-1.58	1.91
0342	polysulfide reductase, subunit A	5.23	1.45
0510-0520	NADH-ubiquinone oxidoreductase	-1.19 - 1.80	-2.07 - 1.20
0529	superoxide dismutase [Fe]	1.78	1.09
0547	heat shock protein Hsp20	-6.4	-1.04
0575	cytochrome c551 peroxidase	11.13	1.65
0667	malate:quinone oxidoreductase	1.19	-1.09
0950	Molybdopterin oxidoreductase	22.22	-
0987	heat shock protein 60 family co-chaperone GroES	1.18	1.48
1334	putative superoxide reductase	3.8	-3.07
1033-1036	lactate utilization proteins ABC	3.72 - 7.61	-3.69 - -3.47
1443	malate dehydrogenase	2.61	1.77
1459	fumarate hydratase class II	3.38	1.04
1679-1680	fumarate hydratase class I	1.34-1.51	-1.96 - -1.59
1703	pyruvate dehydrogenase [ubiquinone]	7.75	1.16
1963	putative heat shock protein HspR	-2.97	-1.48
2521-2523	ubiquinol-cytochrome c reductase	1.7 - 2.01	- 3.08 - -2.26
2652-2654	Cbb3-type cytochrome c oxidase subunit	1.56 - 1.62	- 2.66 - -1.72
3084	superoxide dismutase [Cu-Zn]	2.42	1.23
3225	alkyl hydroperoxide reductase protein C	1.36	6.24
3224	Catalase	-	+*

Supplemental material to:

Tetrachloroethene respiration and proteome analysis of the Epsilonproteobacterium *Sulfurospirillum multivorans* grown in the presence of oxygen

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Mass spectrometric analysis, detailed methods

Separation of tryptic peptides was performed using an Ultimate 3000 nanoRSLC system (Thermo Scientific, Germering, Germany) coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific, San Jose, CA, USA). A sample volume of 3 µL was loaded onto a trapping column with 300 µm inner diameter, packed with 5 µm C18 particles (µ-precolumn, Thermo Scientific, Germany) and separated via a 15 cm analytical column (Acclaim PepMap RSLC, 2 µm C18 particles, Thermo Scientific). The column oven temperature was set to 35°C. During the liquid chromatography (LC) run, a constant flow of 300 nL/min (solvent A: 0.1% formic acid) was applied with a linear gradient of 4% to 55% solvent B (80% acetonitrile, 0.08% formic acid) in 90 min. Mass spectrometer (MS) full scans were performed in the Orbitrap mass analyzer within the mass range of 400-1,700 *m/z* at 60,000 resolution using an automatic gain control target of $4x10^5$ and maximum fill time of 50 ms. The MS analyzed in data-dependent acquisition mode; the highest intense ions with positive charge states between 2 and 7 were selected for MS/MS. An MS/MS isolation window for ions in the quadrupole was set to 1.6 *m/z*. MS/MS scan were acquired within 3 s cycle time (top speed) using the higher energy dissociation mode at a normalized collision induced energy of 35%, a maximum injection time of 120 ms, and a minimum of ion signal threshold for MS/MS of $5x10^4$ counts. The exclusion time to reject masses from repetitive MS/MS fragmentation was set to 30 s. The polysiloxane ion at m/z 445.12003 was used as an internal lock mass.

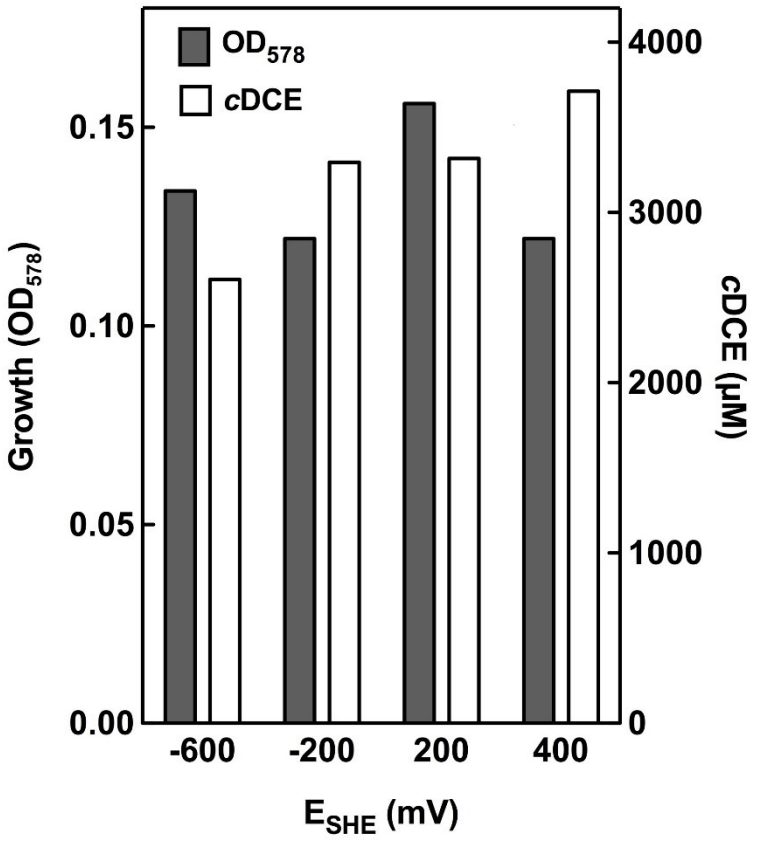


Figure S1: Redox potential-dependent dechlorination of PCE to cDCE in growing *S. multivorans*. The redox potential was adjusted via a potentiostat and measured with a redox electrode (Ag+/AgCl reference electrode with E_{SHE} of +207 mV). *S. multivorans* was cultivated at 28°C under stirring (200 U min-1) for 20h in an anoxic chamber. The medium (40 mM formate, 10 mM PCE, 5 mM acetate) was made anoxic and preincubated at the given potential before inoculation. During the cultivation the redox potential of the medium was adjusted by the potentiostat to the setup potential. After the cultivation OD_{578} and formed *c*DCE were determined.

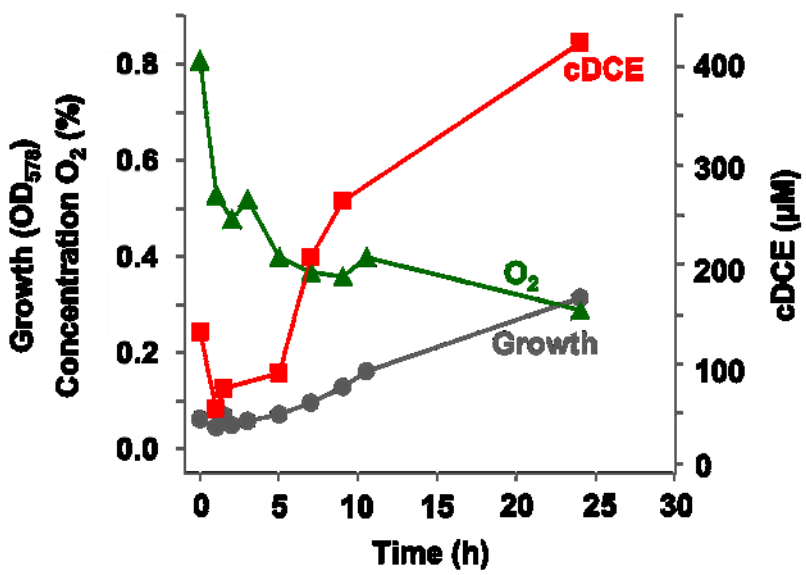


Figure S2: Product (cDCE) formation and growth of *S. multivorans* with pyruvate as electron donor and PCE as electron acceptor in the presence of oxygen (initial concentration in the gas phase 1%). The growth of *S. multivorans* (OD_{578}), the consumption of oxygen as well as the formation of cDCE are shown. 1% O_2 = 0.373 mg/l O_2 at 28°C. The figure is a representative from three independent trials. The initial cDCE was carried over from the PCE dechlorination product of the pre-culture and concentration drops as it is dissolved in the medium.

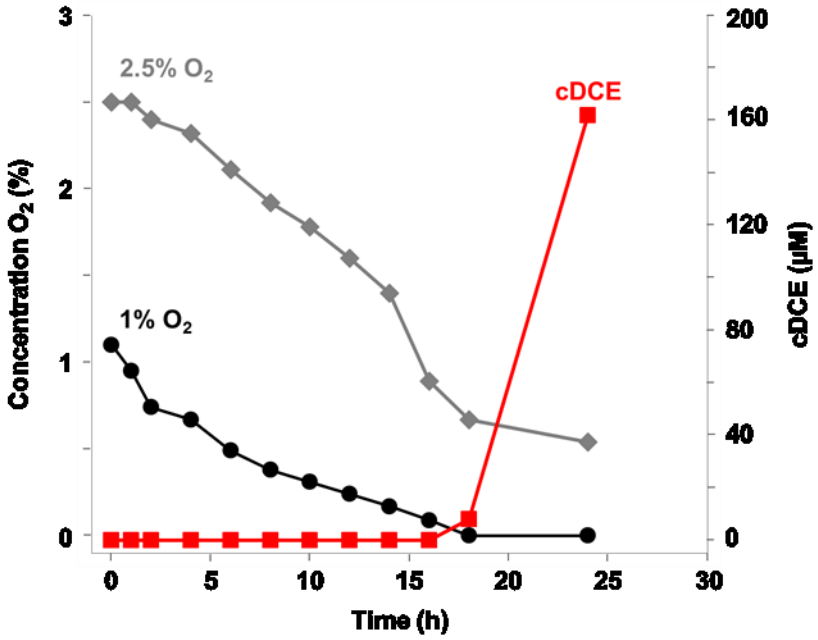


Figure S3: Consumption of oxygen by *D. hafniense* Y51 in cell suspensions containing PCE. Pyruvate served as electron donor, PCE (200 µM) was added to the medium. Formation of cDCE could only be observed when oxygen was depleted completely in the initial presence of 1% oxygen.

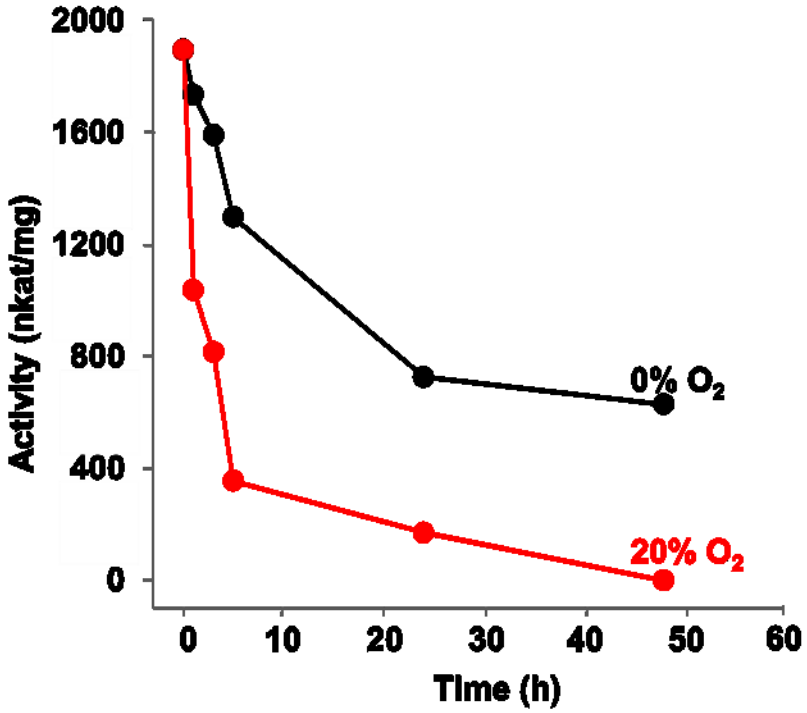
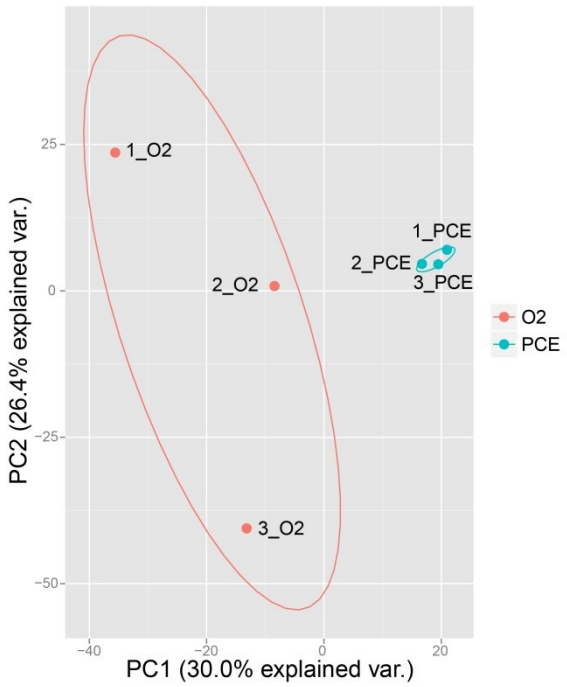
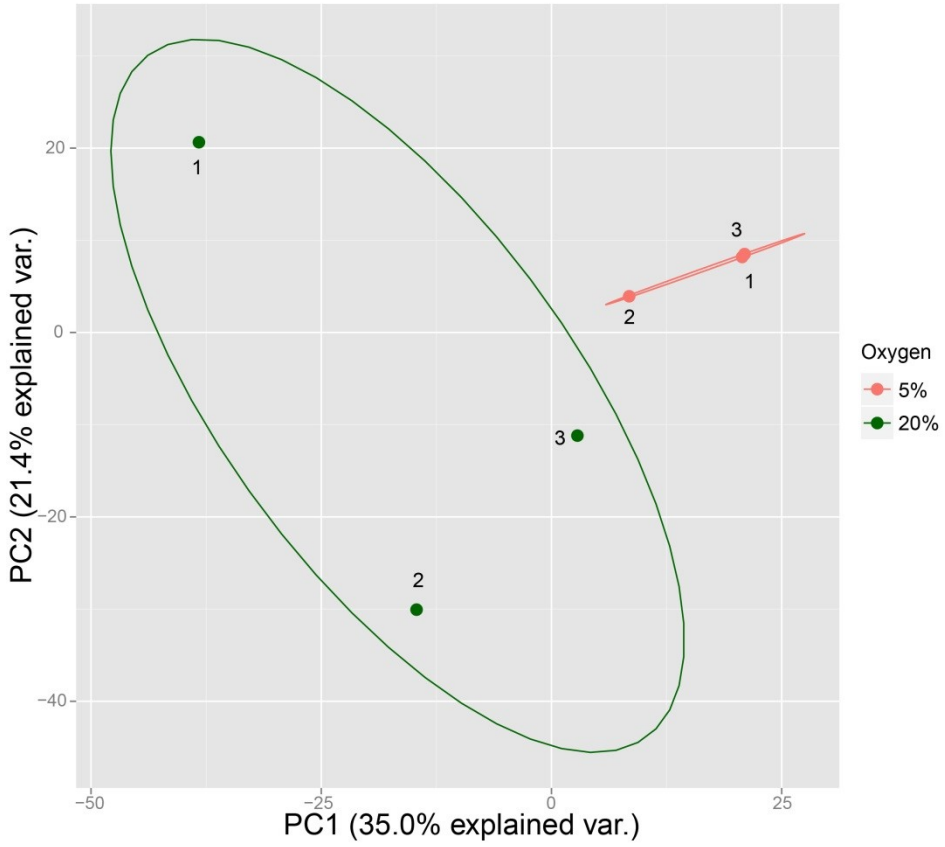


Figure S4: Enzyme-activity of the purified reductive dehalogenase PceA of *S. multivorans* in the presence of different oxygen concentrations. The purified enzyme was incubated at 28°C under stirring and was exposed to the oxygen concentrations indicated. Measurements were carried out in triplicates.

A


1_O2
2_O2
3_O2
1_PCE
2_PCE
3_PCE
O2
PCE
PC2 (26.4% explained var.)
PC1 (30.0% explained var.)
25
0
−25
−50
−40
−20
0
20

B


1
2
3
3
2
1
Oxygen
5%
20%
PC2 (21.4% explained var.)
PC1 (35.0% explained var.)
20
0
−20
−40
−50
−25
0
25

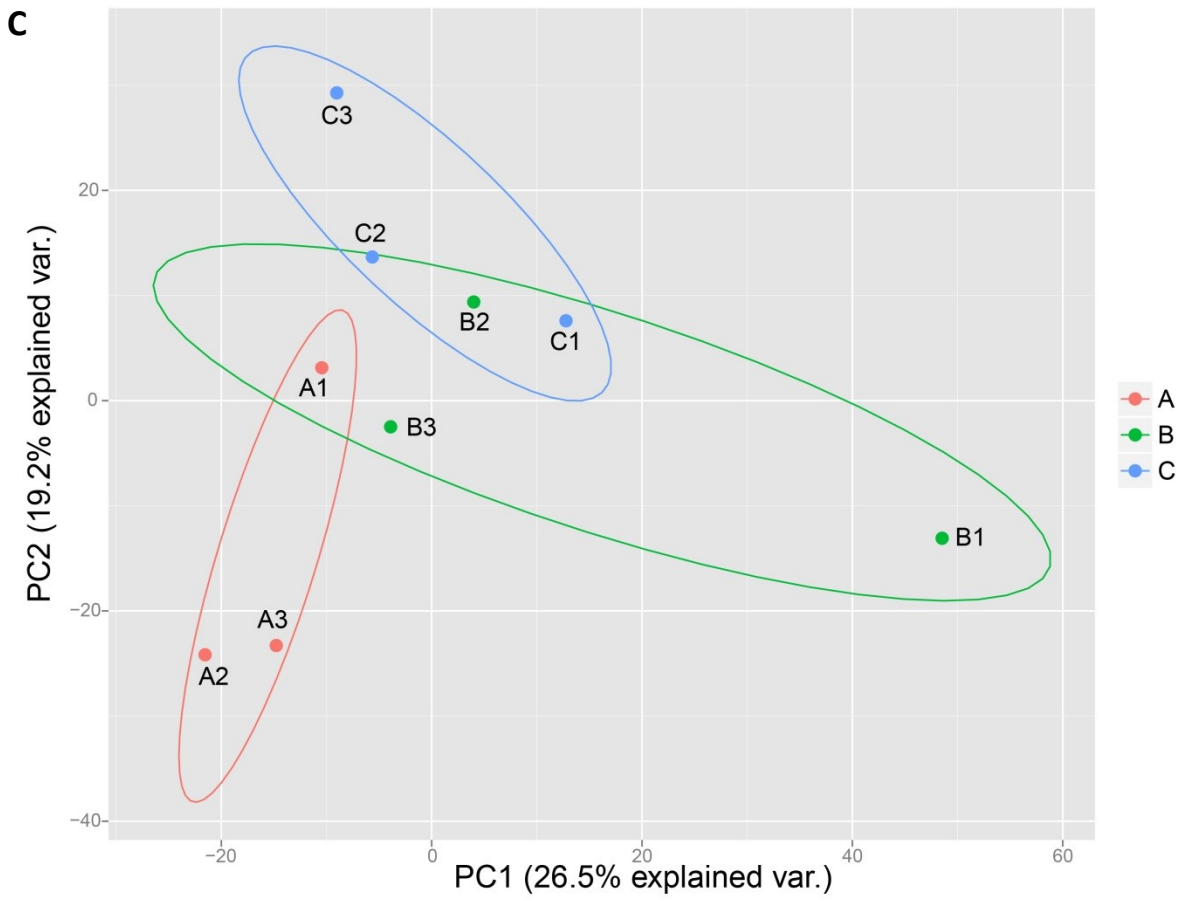


Figure S5: Principle component analyses (PCA) of proteome data. A: PCA of proteome profiles of cells cultivated with PCE as electron acceptor versus cells grown with oxygen as electron acceptor. **B:** PCA of proteome profiles of cells grown with pyruvate/PCE, exposed in exponential phase to 5% or 20% oxygen in the exponential growth phase. **C:** Principle component analysis of proteome profiles of cells induced with PCE in cells after growing with oxygen. A: cells grown on pyruvate/PCE. B: cells grown on pyruvate/PCE plus 5% oxygen; early harvest. C: cells grown on pyruvate/PCE plus 5% oxygen; harvest after induction of PCE respiration. Cultivation conditions are indicated by different colors, biological replicates are numbered.

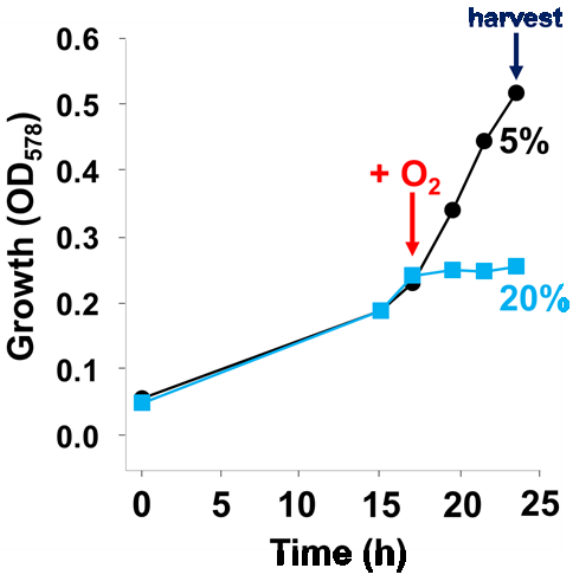


Figure S6: Stress response of *S. multivorans* to atmospheric oxygen concentrations after growing with PCE. Cells were grown until mid-exponential phase when either 5% or 20% oxygen was added to the gas phase. Six and a half hours later, cells were harvested for proteomics.

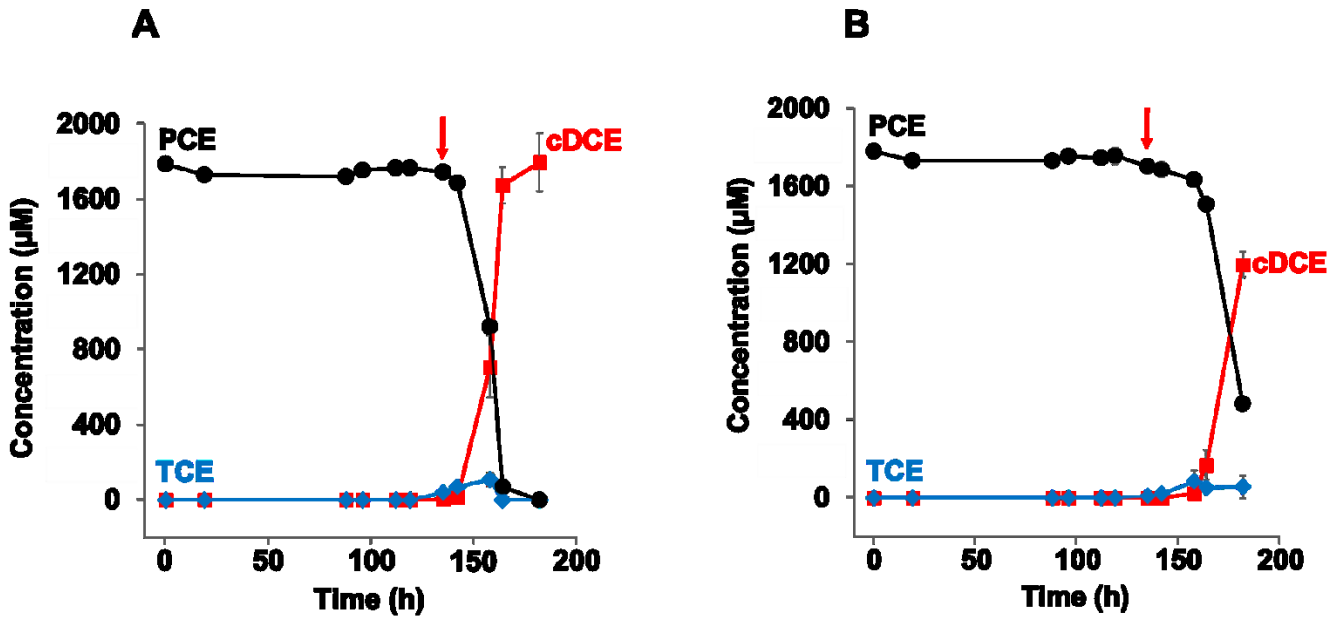


Figure S7: Induction of PCE respiration in the presence of oxygen. The dechlorination of PCE respectively the formation of TCE and cDCE after the induction of PCE respiration in *S. multivorans* cells with down-regulated organohalide respiration is shown. The time point at which PCE dechlorination started is indicated by a red arrow. Pyruvate served as electron donor for all cultures. **A:** Dechlorination of PCE in cells grown with PCE as sole electron acceptor. **B:** Dechlorination of PCE in cells grown with PCE and 5% oxygen as electron acceptors.

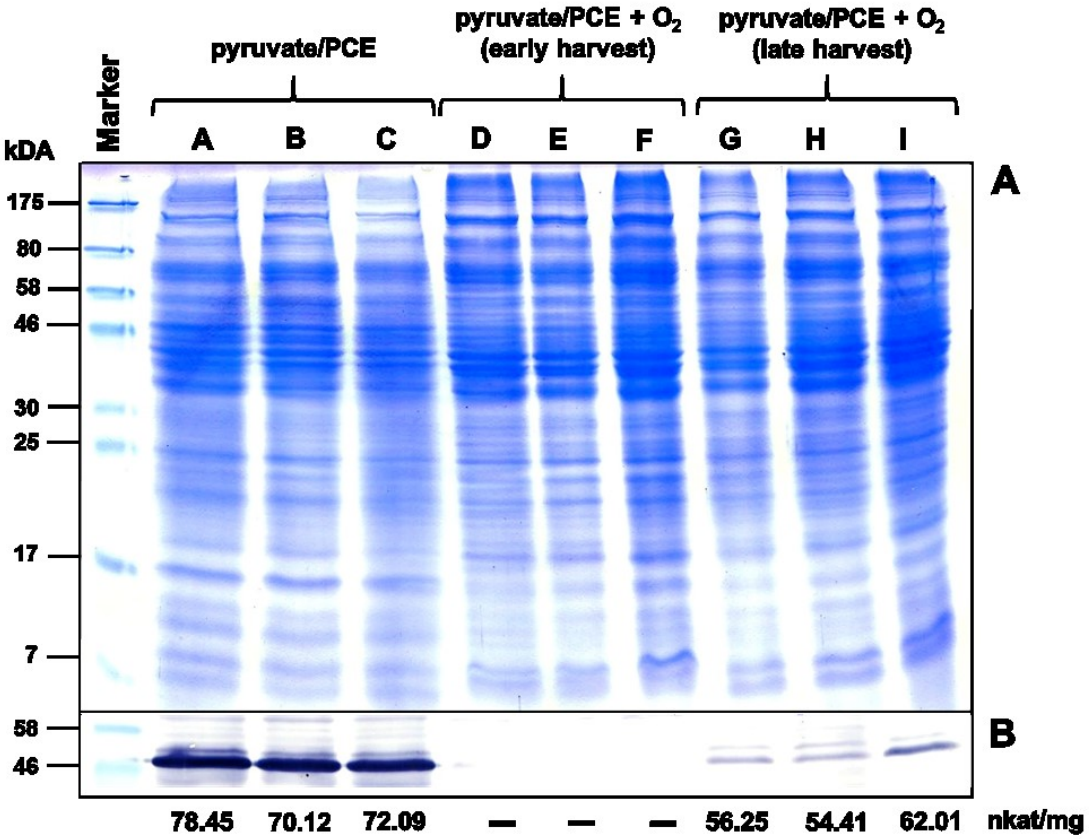


Figure S8: A: Coomassie stained SDS-PAGE: 10 µg protein from all biological replicates which were used in the proteome analysis was applied per lane. **B: Western-Blot: Anti-PceA (1:500.000).** 10 µg protein from all biological replicates which were used in the proteome analysis was applied per lane. PceA is visible at approximately 50kDa. The PceA activities of the crude extracts from all biological replicates investigated are shown.

Supplemental Table S1

Due to the size of the data sets, the supplemental Table S1 was not printed. Here is a brief description of the contents of the single data sheets.

Data sheet 1

This table lists the normalized and logarithmized (log10) data of all single samples.

Data sheet 2

This table includes the differential comparison of the samples within the different experiments.

Data sheets 3 – 6

These tables list the proteins that are up-regulated or down-regulated in the different experiments:

- Data sheet 3: Experiment 1 _ oxygen-grown cells vs. PCE-grown cells
- Data sheet 4: Experiment 2 _ cells exposed to 20% oxygen vs. cells exposed to 5% oxygen
- Data sheet 5: Experiment 3 _ PCE-grown cells vs. PCE + O2-grown cells (early harvest)
- Data sheet 6: Experiment 4 _ PCE-grown cells vs. PCE + O2-grown cells (late harvest)

Data sheet 7

This table lists the proteins of the OHR region, which were detected in the different samples.

4. Diskussion

Identifizierung von Komponenten der PCE-Atmungskette in *S. multivorans*

Organohalid-atmende Bakterien (OHRB) spielen heute bei der biologischen Sanierung von Boden und Grundwasser, die Kontaminationen mit halogenierten organischen Verbindungen aufweisen, eine entscheidende Rolle. Um die OHRB möglichst effektiv für den Prozess der Bioaugmentation einsetzen zu können, ist es daher von besonderer Bedeutung, die grundlegenden Prozesse der reduktiven Dehalogenierung zu verstehen. Während die Schlüsselenzyme der Organohalid-Respiration, die reduktiven Dehalogenasen (RDasen), sowie die Enzyme, die für die Oxidation der Elektronendonoren verantwortlich sind, schon lange bekannt sind, lautet eine zentrale, aber ungelöste Frage in der Organohalid-Atmungsforschung: Welche Proteine und mögliche andere Komponenten (z.B. Chinone) sind an der Elektronenübertragung innerhalb der Organohalid-Atmungsketten beteiligt und wie findet die Energiekonservierung statt? Um der Lösung dieser Frage für das fakultative OHRB *S. multivorans* näher zu kommen, wurden in dieser Arbeit zwei verschiedene Ansätze gewählt: Durch eine Genom- und Proteomanalyse sollten mögliche Proteinkomponenten der PCE-Atmungskette identifiziert und deren mögliche Beteiligung an der Organohalid-Atmung nachgewiesen werden, während durch Inhibitionsversuche mit Zellsuspensionen von *S. multivorans* die Beteiligung von Menachinon und einem revertierten Elektronentransport an der Elektronenübertragung bestätigt werden sollten. Des Weiteren sollten die identifizierten Komponenten isoliert und näher charakterisiert werden.

Genomanalyse

Das Genom von *S. multivorans* ist mit einer Größe von 3.176 Mbp das zweitgrößte vollständig sequenzierte Genom eines Epsilonproteobakteriums, das bislang beschrieben wurde (Goris *et al.*, 2014); das größte ist mit 3.192 Mbp das Genom von *Arcobacter nitrofigilis* (Pati *et al.*, 2010). Im Kontrast dazu weist *S. multivorans* für einen fakultativen OHRB ein relativ kleines Genom auf. Im Allgemeinen haben fakultative OHRB Genome mit Größen zwischen 3.2 und 6.5 Mbp. Im Gegensatz dazu weisen obligate OHRB wie *Dehalococcoides mccartyi* und *Dehalogenimonas lykanthroporepellens*, die bei ihrer Energiegewinnung strikt an die Organohalid-Respiration gebunden sind, sehr kleine Genome mit Größen um die 1.4 Mbp auf (Kruse *et al.*, 2016). Durch einen Vergleich des *S. multivorans* Genoms mit den Genomen anderer *Sulfurospirillum*-Arten konnte eine Genomregion identifiziert werden, in der mögliche Komponenten der PCE-

Atmungskette sowie Proteine für die Regulation der Organhalid-Respiration kodiert sind. Für die Gene in dieser Region finden sich keine Orthologen in den Genomen der nicht-dechlorierenden *Sulfurospirillum*-Vertreter (Goris *et al.*, 2014). In dieser 50 kbp großen Region, die sich nahezu genau gegenüber dem Replikationsursprung befindet, ist auch das Gen lokalisiert, welches für die reduktive Dehalogenase PceA kodiert. Aus diesem Grund wird diese Genomregion, die die Gene SMUL_1516 bis SMUL_1596 umfasst, als OHR-(Organohalid Respiration) Region bezeichnet. Von den Proteinen, die in der OHR-Region kodiert sind, weisen 46 (SMUL_1530 bis SMUL_1575) eine mögliche Verbindung zur Organohalid-Respiration auf und sind in der OHR-Kernregion lokalisiert (Goris *et al.*, 2014; Goris *et al.*, 2015). Die gleiche Region findet sich an ähnlicher Stelle in dem Genom von *S. halorespirans* (Goris *et al.*, 2017) sowie dem bislang unveröffentlichten Genom von *Sulfurospirillum* Spezies SL2 (Goris & Diekert, 2016), die beide zur Organohalid-Respiration befähigt sind (Luijten *et al.*, 2003; Buttet *et al.*, 2013). In all diesen Genomen scheint der Cluster in Bezug auf die Syntenie- und Nukleotidsequenzidentität zu nahezu 100% konserviert zu sein. In den *Sulfurospirillum*-Genomen, die die OHR-Region enthalten, treten Gene, die für das PCE-reduktive Dehalogenase-Operon kodieren, zusammen mit Genen auf, die für ein Zweikomponenten-Regulatorsystem, ein zweites RDase-Operon, eine mutmaßliche Chinol-Dehydrogenase und den Corrinoid-Biosynthese-Gencluster kodieren (Goris & Diekert, 2016). Innerhalb der OHRB ist die OHR-Region von *Sulfurospirillum* spp. einzigartig. In keinem anderen Genom eines OHRB konnte eine solche Ballung an Genen beobachtet werden, die sowohl für direkte Komponenten der Organohalid-Atmungskette und Proteine, die an der Maturation sowie Regulation beteiligt sind als auch für Protein für die Corrinoid-Biosynthese, kodieren. Auch bei anderen Atmungsenzymen, wie zum Beispiel der Nitrat-Reduktase von *W. succinogenes*, kann eine Konzentration von funktionell verknüpften Genen beobachtet werden. Die Nitratreduktase wird durch das erste Gen im napAGHBFLD-Gencluster kodiert (Kern & Simon, 2009). Zusammen mit dem Cytochrom *c*-haltigem NapB Protein bildet sie ein Heterodimer aus. Daneben finden sich in dem Cluster noch Gene, die für eine Chinol-Dehydrogenase kodieren (*napGH*) sowie Gene, die an der Maturation *(napD* und *napF*) von NapA beteiligt sind (Kern *et al.*, 2007). Damit spiegelt das napAGHBFLD-Gencluster zum Teil die Colokalisierung von funktionell verknüpften Genen wieder, wie sie in der OHR-Kernregion zu finden ist. Auch in anderen OHRB werden *rdhAB*-Operons regelmäßig von zusätzlichen Genen begleitet, wobei dies in viel geringerem Ausmaß geschieht als in der OHR-Kernregion beobachtet. Die Funktion der meisten dieser akzessorischen Gene ist bis heute unbekannt (Kruse *et al.*, 2016). Einige der Gene kodieren für Proteine, die an der Regulation der Expression der *rdhAB*-Gene beteiligt sind (Wagner *et al.*, 2013; Gábor *et al.*, 2008; Pop *et al.*, 2004), während andere als Chaperone fungieren, die wahrscheinlich bei der Faltung der Dehalogenasen eine Rolle spielen (Maillard *et al.*, 2011; Mac Nelly *et al.*, 2014; Morita *et al.*,

2009). Ungewöhnlich ist in *S. multivorans* die enge Verknüpfung des *pceA* Gens mit Genen, die für die Kofaktorbiosynthese kodieren. Im Gegensatz dazu sind in dem PCE-dechlorierenden Organismus *Desulfitobacterium hafniense* Y51, der zur de novo Corrinoid-Synthese befähigt ist (Reinhold *et al.*, 2012), nahezu alle Gene für die Corrinoid-Biosynthese und –Aufnahme an drei genomischen Loci lokalisiert und nicht direkt verknüpft mit den RDase-Genen (Nonanka *et al.*, 2006). Auch in anderen OHRB, wie zum Beispiel *D. hafniense* DCB-2 oder *D. hafniense* TCE1 (Kim *et al.*, 2012; Choudhary *et al.*, 2013) und *Geobacter lovleyi* SZ (Wagner *et al.*, 2012), konnte keine Clusterbildung zwischen Genen der Corrinoid-Biosynthese und Genen für reduktive Dehalogenasen beobachtet werden.

Proteomanalyse

Für verschiedene Enzyme wurde die Induktion der Genexpression durch ihr Substrat bereits beschrieben. Gut untersucht ist das *lac*-Operon von *Escherichia coli*, dessen Expression durch Laktose, dem Substrat der β-Galaktosidase, codiert im *lac*-Operon, induziert wird. Ein weiteres Beispiel, für das die Regulation der Genaktivität durch Substratinduktion beschrieben ist, sind die membrangebundene (codiert durch das *narGHJI*-Operon) und die periplasmatische (codiert durch das *napFDAGHBC*-Operon) Nitrat-Reduktase aus *E. coli*. Die Expression der beiden unterschiedlichen Operons erfolgt hier in Abhängigkeit von der Konzentration des Nitrats (Wang *et al.*, 1999). Auch für die unterschiedlichen reduktiven Dehalogenasen der OHRB konnte eine Substrat-abhängige Genexpression beobachtet werden (Kruse *et al.*, 2016). So konnten durch Transkriptanalysen in Anwesenheit von spezifischen Substraten zum Beispiel die BvcA, die Vinylchlorid-RDAse aus *D. mccartyi* Stamm BAV1 (Krajmalnik-Brown *et al.*, 2004), die DcpA, die 1,2-Dichlorpropen-Dichlor-elemenierende RDase aus *Dehalogenimonas lykanthroporepellens* und nicht identifizierter *D. mccartyi* Stämme aus zwei Anreicherungskulturen (Padilla-Crespo *et al.*, 2014) sowie RdhA1, verantwortlich für die Dichlor-Eleminierung von 1,2-Dichlorethan durch einen *Dehalobacter* Stamm (Groster & Edwards, 2009) identifiziert werden. Damit stellen Transkriptanalysen wie auch Proteomanalysen in Anwesenheit spezifischer Organohalide ein wichtiges Werkzeug zur Identifizierung möglicher RDasen sowie weiterer Proteine beteiligt an den Organohalid-Respiration der unterschiedlichen OHRB dar. Dies gilt insbesondere, da die OHRB - bis auf wenige Ausnahmen - genetisch nicht zugänglich sind und die Beteiligung von Enzymen an der Organohalid-Atmung dieser Organismen nicht durch das Entfernen der entsprechenden Gene untersucht werden kann. Für *Desulfitobacterium dehalogenans* JW/IU-DC1 wurde eine Transposonmutagenese durch Tn*916* sowie eine Genunterbrechung durch die Verwendung eines

thermosensitiven Plamids beschrieben (Smidt *et al.*, 1999, 2001). In das Genom von *S. multivorans* wurde ein vollständiger Vektor, der ein modifiziertes *pceAB*-Operon enthielt, integriert. Das modifizierte *pceAB*-Operon enthielt ein *pceA*-Gen, an dessen C-Terminus ein Strep-Tag kloniert wurde (Bommer *et al.*, 2014). In dieser Arbeit konnten *pceA* sowie weitere Gene lokalisiert in der OHR-Kernregion von *S. multivorans* nicht deletiert werden (Daten nicht gezeigt). Aus diesem Grund wurde mit einer umfassenden vergleichenden Proteomanalyse ein indirekterer Ansatz gewählt um die Beteiligung von Proteinen der OHR-Kernregion an der PCE-Atmung von *S. multivorans* zu zeigen.

Für die gesamte OHR-Kernregion konnte eine PCE-abhängige Regulation wie sie bereits für PceA beschrieben wurde (John *et al.*, 2009) beobachtet werden (Goris *et al.*, 2015), was die Annahme stützt, dass die in dieser Region lokalisierten Gene für Proteine kodieren, die entweder direkt oder indirekt an der PCE-Atmung beteiligt sind. Dabei erfolgt die Regulation auf transkriptioneller Ebene. Bei langer Kultivierung von *S. multivorans* in Abwesenheit von PCE kommt es zu einer langsamen Herunterregulierung der Transkriptmengen (Daten nicht gezeigt), wobei durch erneute Kultivierung in Anwesenheit von PCE die Produktion der Proteine aus der OHR-Kernregion wieder induziert werden kann (Manuskript VI).

Bislang ist wenig darüber bekannt wie die Regulation der Organohalid-Atmung in den verschiedenen OHRB erfolgt. Ausgehend von den vorhandenen Genomsequenzen scheint es, dass sich die Regulations-Mechanismen zwischen den einzelnen phylogenetischen Gruppen der OHRB unterscheiden und weniger mit der Stoffwechselleitung der Organismen - fakultativer oder obligater OHRB - zusammenhängen (Kruse *et al.*, 2016). Mögliche Kandidaten für die Regulation der Organohalid-Respiration in *S. multivorans* sind zwei Zweikomponenten-Regulatorsysteme, die jeweils stromabwärts der *rdh*-Operons lokalisiert sind. Jedes der Systeme besteht aus einer putativen Histidin-Protein-Kinase (HPK; SMUL_1534 und SMUL_1538) sowie einem putativen Response-Regulator (RR; SMUL_1535 und SMUL_1539). Als wahrscheinlichster Kandidat für die Regulation der Organohalid-Atmung wurde das RR Protein SMUL_1539 identifiziert. Es konnte in allen *S. multivorans* Proben unabhängig vom verwendeten Elektronenakzeptor in etwa gleichen Mengen detektiert werden. Auch konnte bei einer Transkript-Analyse des für SMUL_1539 kodierenden Gens keine Veränderung der Transkriptmenge in Abhängigkeit von der Anwesenheit bzw. Abwesenheit von PCE als Elektronenakzeptor beobachtet werden (Daten nicht gezeigt). Diese Ergebnisse stützen die wahrscheinliche Funktion von SMUL_1539 - die Wahrnehmung von PCE – als eine Fähigkeit, die insbesondere in Abwesenheit von PCE aufrechterhalten werden muss (Goris *et al.*, 2015). Weitere OHRB, in denen Zweikomponenten-Regulatorsysteme möglicher Weise an der Regulation der Organohalid-Respiration beteiligt sind, sind *Dehalococcoides* spp.. In *Dehalococcoides* spp. treten die *rdhA*-Gene meist assoziiert mit Genen,

die entweder für Zweikomponenten-Regulatorsysteme oder für MarR-Regulatoren (multiple antibiotic resistance regulator) kodieren, auf (Kruse *et al.*, 2016). Im Gegensatz zur Situation in *S. multivorans,* wo für beide Histidin-Protein-Kinasen 7 Transmembranhelices vorhergesagt wurden (Goris *et al.*, 2014), scheinen die Histidinkinase-Komponenten der Zweikomponenten-Regulatorsysteme in *Dehalococcoides* spp. jedoch zytoplasmatisch lokalisiert zu sein, da für sie keine Transmembranhelices vorhergesagt wurden (Kruse *et al.*, 2016). Auch bei den MarR-Regulatoren handelt es sich um lösliche Proteine.

Beteiligung einer putativen Chinol-Dehydrogenase an der PCE-Atmung in *S. multivorans*

Eine putative Chinol-Dehydrogenase, bestehend aus einer membranintegralen Komponente (SMUL_1542) sowie einer periplasmatisch lokalisierten Komponente (SMUL_1541), wurde als wahrscheinliche Komponente identifiziert, die an der Elektronenübertragung innerhalb der PCE-Atmungskette von *S. multivorans* beteiligt ist (Goris *et al.*, 2014; Goris *et al.*, 2015). Die putative Chinol-Dehydrogenase zeigt Ähnlichkeiten zu der NapGH- und NosGH-Chinol-Dehydrogenase aus *Wolinella succinogenes*. Die NapGH-Chinol-Dehydrogenase wurde als Komponente, verantwortlich für die Chinoloxidation und den Elektronentransport auf die terminale Reduktase NapA (über das essentielle periplasmatische Dihäm-Cytochrom *c* NapB) in der Nitrat-Atmung, beschrieben (Kern *et al.*, 2008) (Abbildung 1-1). Durch Deletionsmutanten konnte die essentielle Bedeutung von NapG und NapH für die Nitratatmung von *W. succinogenes* gezeigt werden (Kern *et al.*, 2008), wobei die postulierte Chinon-Bindestelle in NapH (Simon *et al.*, 2008) bislang noch nicht experimentell nachgewiesen werden konnte. *Wolinella succinogenes*, obwohl kein Denitrifizierer, ist in der Lage N_2O zu reduzieren (Teraguchi & Hollocher, 1989) und auch durch Formiat-abhängige N_2O-Reduktion zu wachsen (Yoshinari, 1980; Payne *et al.*, 1982; Costa *et al.*, 1990). Die terminale Reduktase ist die Distickstoffmonoxid-Reduktase *c*NosZ. Bislang wurde angenommen, dass die Proteine NosG und NosH eine NapGH-ähnliche Chinol-Dehydrogenase bilden und am Elektronentransport vom Menachinon-Pool zu *c*NosZ beteiligt sind (Simon *et al.*, 2004). Kürzlich konnte durch Deletionsmutanten, die essentielle Rolle von NosGH für die N_2O-Reduktion gezeigt werden. Weitere Komponenten, die als essentiell für die N_2O-Reduktion identifiziert wurden, waren die Häm-enthaltenden Untereinheiten (QcrB und QcrC) des Cytochrom *bc*1-Komplex (persönliche Mitteilung Prof. Dr. Simon, TU Darmstadt). Auf Grund der Ergebnisse wird vermutet, dass NosGH nicht direkt an der Chinoloxidation beteiligt ist, sondern zusammen mit dem Cytochrom *bc*1-Komplex, an dem vermutlich die Chinoloxidation stattfindet, einen größeren Komplex bildet, der evtl. als Protonenpumpe fungiert. Es gilt zu verifizieren, ob für die

putative Chinol-Dehydrogenase NapGH von *W. succinogenes* das Gleiche gilt wie für NosGH. Auch für die putative Chinol-Dehydrogenase SMUL_1541/1542 von *S. multivorans* muss untersucht werden, ob sie direkt an der Chinoloxidation beteiligt ist oder ob es auch hier zur Ausbildung eines größeren Komplexes entweder mit dem Cytochrom *bc*1-Komplex oder einer bisher noch unbekannten Komponente kommt (Abbildung 4-1). Eine mögliche Beteiligung des Cytochrom *bc*1-Komplexes am Elektronentransfer in der Organohalid-Respiration könnte durch Hemmversuche mit Myxothiazol untersucht werden. Myxothiazol ist ein spezifischer Inhibitor, der die Elektronenweiterleitung innerhalb des Cytochrom *bc1*-Komplexes hemmt (Berry *et al.*, 2009; Deeudom *et al.*, 2006). Der erfolgreiche Einsatz von Myxothiazol an ganzen Zellen konnte bereits für die Sauerstoff- und Nitrit-Atmung von *Neisseria meningitidis* gezeigt werden (Deeudom *et al.*, 2006). Die Elektronenübertragung von dem periplasmatisch lokalisiertem Fe/S- Protein SMUL_1541 auf die reduktive Dehalogenase PceA ist als sehr wahrscheinlich anzusehen. In *W. succinogenes* konnte gezeigt werden, dass NapG eine spezifische Funktion in der Übertragung von Elektronen auf NapAB ausübt (Kern & Simon, 2008). In dieser Arbeit wurde gezeigt, dass SMUL_1541 gereinigt werden kann (Manuskript V). Interaktionsversuche mit der reduktiven Dehalogenase PceA, die ebenfalls gereinigt werden kann (Neumann *et al.*, 1996; Bommer *et al.*, 2014), wären eine Möglichkeit mehr über den Elektronentransfer zwischen diesen beiden Enzymen zu lernen. In einem ersten Schritt könnte zum Beispiel durch Gel-Shift-Experimente überprüft werden, ob es zur Ausbildung eines SMUL_1541/PceA-Komplexes kommt, was für eine direkte Elektronenübertragung zwischen den Komplexen sprechen würde.

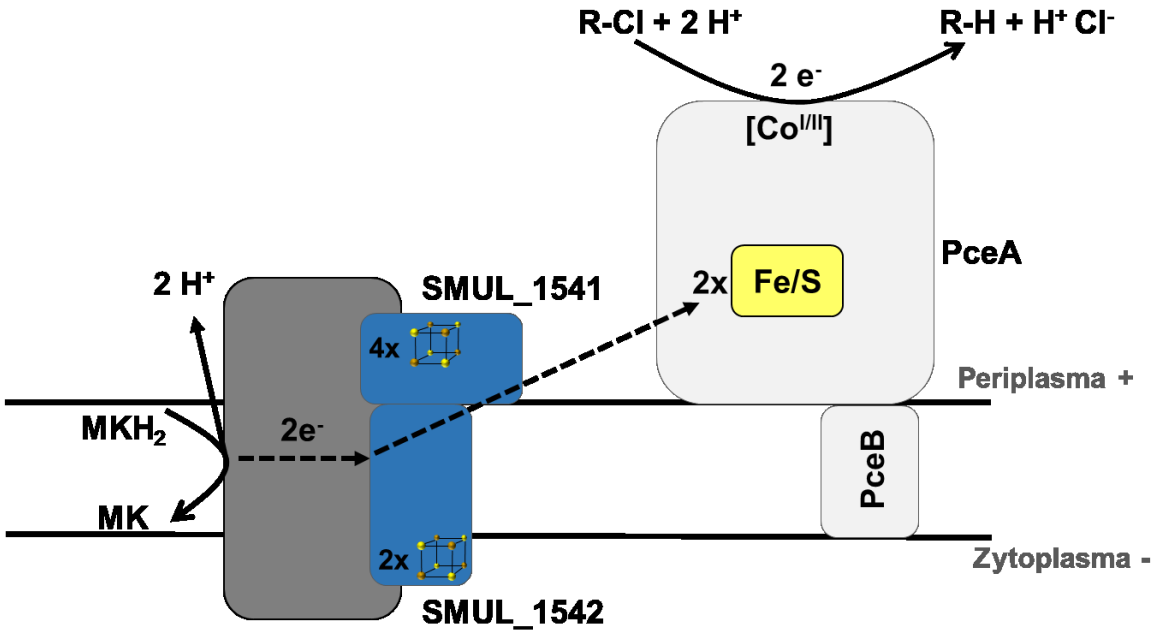


Abbildung 4-1: Hypothetischer Elektronentransferweg von Menachinol zur reduktiven Dehalogenase PceA. Die gestrichelten Pfeile stellen den möglichen Elektronentransferweg über einen Multienzymkomplex bestehend aus SMUL_1541/SMUL_1542 und einem bislang nicht identifizierten Enzym dar. Die Oxidation des Menachinols (MKH_2) erfolgt durch das unbekannte Enzym.

Die einzige andere putative Chinol-Dehydrogenase, die an der Organohalid-Respiration beteiligt sein könnte, ist in *Desulfomonile tiedjei*, einem 3-Chlorobenzoat-atmendem Gammaproteobakterium, kodiert (Goris *et al.*, 2014). Eine weitere mögliche Komponente, beteiligt am Elektronentransport in der Organohalid-Respiration, ist das Protein C (Abbildung 4-2B) (Schubert & Diekert, 2016). Die Gene, die für Protein C kodieren, finden sich in einer kleinen Anzahl von RDase-Operons, wie zum Beispiel in *Desulfitobacterium hafniense* Stamm TCE1 und Stamm Y51 (Maillard *et al.*, 2005; Nonaka *et al.*, 2006). Obwohl das C Protein Sequenzähnlichkeiten mit den membranintegralen Regulatorproteinen vom NirI/NosR-Typ aufweist (Saunders *et al.*, 1999; Wunsch & Zumft, 2005), kann eine Beteiligung des C Proteins an der Elektronenübertragung zur RDase nicht ausgeschlossen werden. In der N-terminalen Hälfte des C Proteins findet sich eine nicht-membranintegrale, putative FMN-Bindedomäne, die einen Flavin-Kofaktor enthalten könnte (Abbildung 4-2B). Dieser Kofaktor könnte an der Elektronenübertragung beteiligt sein (Schubert & Diekert, 2016). Der membranintegrale, C-terminale Teil des Proteins weist Ähnlichkeiten zum Membranprotein NapH aus *W. succinognes* auf. Diese Ähnlichkeit könnte auf eine ähnliche Funktion des C Proteins hinweisen, allerdings fehlen im C Protein die Fe/S Cluster-Bindemotive, die in NapH zu finden sind (Schubert & Diekert,

2016). Auch hier wäre eine mögliche Beteiligung des Cytochrom *bc*1-Komplexes am Elektronentransport innerhalb der Organohalid-Atmungskette zu prüfen.

Beteiligung von Menachinon an der PCE-Atmung von *S. multivorans*

Dienen Wasserstoff oder Formiat als Elektronendonoren in der PCE-Atmung von *S. multivorans*, sind sowohl die Elektronendonor-Oxidoreduktasen (Hydrogenase bzw. Formiat-Dehydrogenase) als auch die reduktive Dehalogenase PceA auf der periplasmatischen Seite der Zytoplasmamembran lokalisiert. Damit ist die Möglichkeit, Protonenmotorische Kraft (PMK) über eine skalare Protonenfreisetzung zu erzeugen, nicht gegeben. Die PMK muss über Mechanismen wie Protonenpumpen oder Redoxschleifen aufgebaut werden (John *et al.*, 2006). Dabei kann die Protonentranslokation grundsätzlich mit zwei Reaktionen dieser kurzen Elektronentransportkette verknüpft sein: Mit der Oxidation des Elektronendonors Wasserstoff bzw. Formiat oder mit der Reduktion von PCE an der reduktiven Dehalogenase PceA (Fincker & Spormann, 2017).

Schon früh wurde die Beteiligung von Menachinon an der Elektronenübertragung innerhalb der Organohalid-Respiration von *S. multivorans* postuliert. Aus *S. multivorans* konnten verschiedene Menachinone extrahiert werden (Neumann *et al.*, 1995). In dieser Arbeit wurde durch die Extraktion von Menachinon aus Zellen, gewachsen auf verschiedenen Substratkombinationen, gezeigt, dass *S. multivorans* Menachinon-6 und Methylmenachinon-6 enthält (Manuskript IV). Beide Menachinone, die in *S. multivorans* detektiert wurden, finden sich ebenfalls in dem nicht-dechlorierenden Stamm EK7, der eine Art innerhalb der Gattung *Sulfurospirillum* ist (Ballerstedt *et al.*, 2004). In dem obligaten OHRB *Dehalobacter restrictus* PER-K23 wurden Menachinon-6 neben Menachinon-7, Menachinon-8 und Menachinon-9 gefunden (Maillard & Holliger, 2016). Die Beteiligung von Menachinon an der Organohalid-Atmung von *S. multivorans* wurde durch das Chinon-Analogon 2-n-Heptyl-4-Hydroxyquinolin N-oxid (HQNO) gezeigt (Manuskript IV). Andere OHRB, für die eine Menachinon-abhängige Organohalid-Respiration beschrieben wurde, sind *D. restrictus* (Schumacher & Holliger, 1996), *D. tiedjei* (Louie & Mohn, 1999) und *D. dehalogenans* (Kruse *et al.*, 2015). Zusammengenommen weisen diese Beobachtungen auf die Beteiligung von Chinonen an den Organohalid-Atmungsketten bestimmter fakultativer OHRB hin, in denen die Energiekonservierung nicht an den RDasen erfolgt, sondern stattdessen bei der Übertragung von Elektronen auf Chinone, beispielsweise über einen Komplex I oder von einer Hydrogenase (Abbildung 4-2A/B) (Fincker & Spormann, 2017).
Der obligate OHRB *D. mccartyi* ist bei seiner Energiegewinnung auf die Organohalid-Respiration beschränkt. Daher würde das Vorkommen oder Fehlen von Chinonen und/oder der Gene, die für

an der Chinon-Biosynthese beteiligten Proteine kodieren, ein starker Hinweis für oder gegen die Beteiligung von Chinonen an der Elektronenübertragung in der Organohalid-Atmungskette dieser Organismen darstellen. Ein vollständiger Chinon-Biosyntheseweg wurde bislang in keinem der sequenzierten *D. mccartyi* Genome gefunden (Schipp *et al.*, 2013). Extrahiert wurden Chinone aus *D. mccartyi* Stamm BAV1 und FL2 (White *et al.*, 2005), wobei ihre Quelle unbekannt ist. Die Wasserstoff-getriebene reduktive Dehalogenierung von 1,2,3-Trichlorbenzen durch *D. mccartyi* CBDB1 wurde in Anwesenheit von HQNO nicht gehemmt (Jayachandran *et al.*, 2004). Diese Beobachtungen unterstützen die Hypothese von einem Chinon-unabhängigen Elektronentransport in der Organohalid-Atmungskette von *D. mccartyi*. In *D. mccartyi* wäre damit eine direkte Interaktion der Elektronen-liefernden Oxidoreduktasen mit RDasen denkbar (Abbildung 4-2C). Durch die Bestimmung der Protein-Abundanz in verschiedenen *D. mccartyi* Stämmen wurden erste Hinweise bezüglich der Elektronen-liefernden Oxidoreduktasen, beteilig an der Organohalid-Respiration, gewonnen (Adrian *et al.*, 2007; Morris *et al.*, 2007). Unter den meist gefundenen Proteinen waren eine membrangebundene [NiFe]-Hydrogenase sowie eine putative Formiat-Dehydrogenase. Da für die putative Formiat-Dehydrogenase keine entsprechende Enzymaktivität gezeigt werden konnte, wurde sie im Folgenden als komplexes Eisen-Schwefel-Molybdoenzym (CISM) I bezeichnet (Rothery *et al.*, 2008). Für *D. mccartyi* CBDB1 wurde die Bildung eines Multienzymkomplexes, bestehend aus der membrangebundenen Hydrogenase, CISM I und reduktiver Dehalogenase beschrieben (Kublik *et al.*, 2016; Hartwig *et al.*, 2017). Die Energiekonservierung durch das Pumpen von Elektronen könnte in der Chinon-unabhängigen Organohalid-Respiration an den Transfer von Elektronen von der Hydrogenase auf den CISM-Komplex gekoppelt sein (Zinder, 2016; Pinske *et al.*, 2015).

Beteiligung eines revertierten Elektronentransports an der PCE-Atmung von *S. multivorans*

PCE und TCE stellen auf Grund ihrer positiven Redoxpotentiale ($E^{0'}$(PCE/TCE) = +580 mV; $E^{0'}$(TCE/cDCE) = +540 mV) ideale Elektronenakzeptoren für die Organohalid-Respiration dar. Eine Elektronenübertragung von Menachinon, das ein Standard-Redoxpotential von E_{SHE} = -74 mV (bei pH 7,0) aufweist, auf PCE oder TCE wäre damit thermodynamisch günstig. Allerdings wurde der [Co^{I}]-Zustand (E_{SHE} = -380 mV) des Corrinoid-Kofaktors von PceA als reaktive Spezies identifiziert, die das Organohalid angreift (Schubert & Diekert, 2016). Zudem sind die Redoxpotentiale der zwei Fe/S Cluster, die höchst wahrscheinlich für die Übertragung der Elektronen auf den Corrinoid-Kofaktor verantwortlich sind, sogar noch negativer (E_{SHE} = -450 mV; Siritanaratkul *et al.*, 2016). Zwei Mechanismen stehen im Vordergrund wenn es darum geht, die

Erzeugung von Elektronen mit niedrigem Redoxpotenial aus Chinolen zu erklären: Ein revertierter Elektronentransport, abhängig von einer Protonenmotorischen Kraft über die Membran und die Elektronenbifurkation. In der kürzlich entdeckten Flavin-basierten Elektronenbifurkation wird die endergonische Aktivierung von Elektronen durch eine begleitende exergonische Redoxreaktion angetrieben (Buckel & Thauer, 2013). Es wurde gezeigt, dass diese Art der Elektronenaktivierung am Katabolismus verschiedener, streng anaerober Mikroorganismen beteiligt ist.

In *S. multivorans* wird die thermodynamisch ungünstige Reduktion von [Co^{I}] zu [Co^{II}] durch einen revertierten Elektronentransport (r.e.f.) angetrieben (Abbildung 3-2A). In dieser Arbeit (Kapitel 2.4) wurde gezeigt, dass in Zellsuspensionen von *S. multivorans* die PCE-Atmung bei Verwendung von Wasserstoff oder Formiat als Elektronendonoren durch den Einsatz von Protonophoren wie Carbonyl Cyanid-*p*-trifluoromethoxyphenylhydrazon (FCCP; 15 nmol/mg Protein) vollständig gehemmt werden kann. Damit wurden frühere Beobachtungen bestätigt (Miller *et al.*, 1996). Die Fumarat-Atmung blieb unter diesen Bedingungen unbeeinflusst (Kapitel 2.4; Miller *et al.*, 1996). Auch in *Sulfurospirillum halorespirans* konnte die PCE-Atmung durch den Einsatz von FCCP (15 nmol/mg Protein) vollständig gehemmt werden. Dieses Ergebnis spricht dafür, dass auch in dieser *Sulfurospirillum* Art ein revertierter Elektronentransport Bestandteil der Organohalid-Atmungskette ist. In Zellsuspensionen von *D. hafniense* Y51 konnte die PCE-Atmung durch den Einsatz von Protonophoren nicht gehemmt werden, was mit großer Wahrscheinlichkeit gegen die Beteiligung eines revertierten Elektronentransports an der Organohalid-Atmung in diesem Organismus spricht. Ein weiterer Organismus, dessen PCE-Atmung nicht durch Protonophore beeinflusst wird, ist *D. restrictus*. Es wurde gezeigt, dass H_2-reduzierte Zellen von *D. restrictus* PCE und TCE in Anwesenheit des Protonophors Carbonyl Cyanid-*m*-chlorophenylhydrazon (CCCP; 15 nmol/mg Protein) effizient dechlorieren (Schumacher & Holliger, 1996). Auch hier ist die Beteiligung einer revertierten Elektronentransports an der Organohalid-Respiration damit sehr unwahrscheinlich.

Ausgehend von den Ergebnissen dieser Arbeit und den in der Literatur beschriebenen Beobachtungen kann folgende Annahme getroffen werden: Die Organohalid-Atmungsketten der verschiedenen OHRB unterscheiden sich sowohl in Bezug auf die Beteiligung von Menachinon sowie weiterer Komponenten (putative Chinol-Dehydrogenasen, C Protein) an der Elektronenübertragung innerhalb der Atmungsketten als auch in der Beteiligung eines revertierten Elektronentransports an der Organohalid-Atmung.

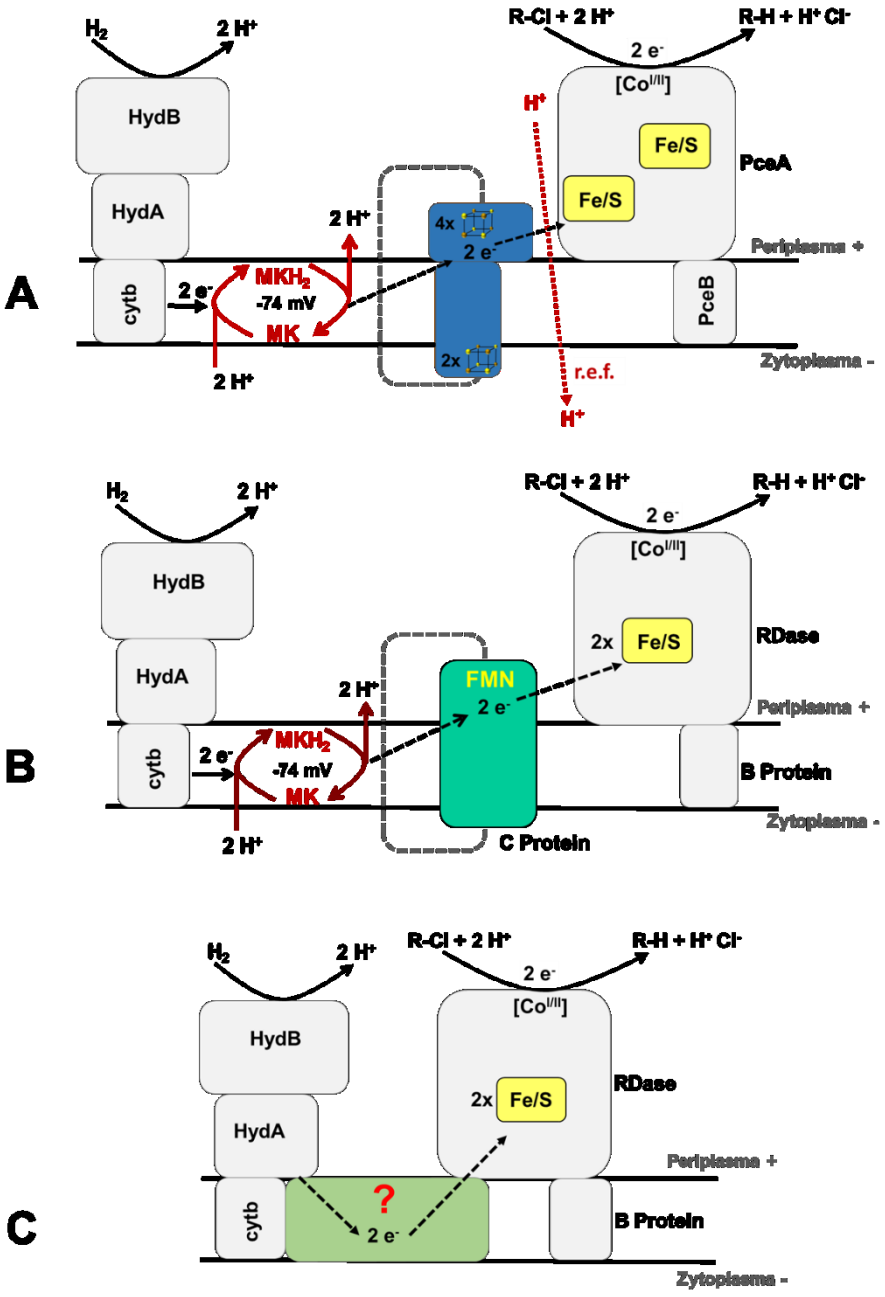


Abbildung 4-2: Vorläufige Modelle verschiedener Organohalid-Atmungsketten mit Wasserstoff als Elektronendonor. **A:** Modell der PCE-Atmungskette von *S. multivorans* mit Menachinon und einer putativen Chinol-Dehydrogenase als Elektronenüberträger innerhalb der Atmungskette. **B:** Modell einer menachinon-abhängigen Organohalid-Atmungskette in Organismen, die das C Protein kodieren. In Modell A und B erfolgt die Energiekonservierung durch die Reduktion des Menachinons durch die Hydrogenase. Bei der Reduktion des Menachinons werden Protonen aus dem Zytoplasma aufgenommen. Die Oxidation des Menachinols erfolgt entweder durch die putative Chinol-Dehydrogenase (A), das C Protein (B) oder einen bislang unbekannten Elektronenüberträger (angedeutet durch das gestrichelt dargestellte Protein). **C:** Modell einer Chinon-unabhängigen Organohalid-Atmungskette. MK/MKH2: Menachinon/Menachinol; r.e.f.: revertierter Elektronentransport.

Reduktive Dechlorierung von PCE unter mikroaeroben Bedingungen

Für die erfolgreiche Bioremediation von Standorten, kontaminiert mit halogenierten organischen Verbindungen, ist es nicht nur von entscheidender Bedeutung, die grundlegenden Prozesse der reduktiven Dehalogenierung zu verstehen, sondern auch zu untersuchen, unter welchen Bedingungen die OHRB tatsächlich in der Lage sind, Organohalide in der Umwelt abzubauen. Für PCE wurde gezeigt, dass die mikrobielle Dechlorierung der Verbindung unter anoxischen Bedingungen bei Umgebungspotentialen kleiner als -180 mV erfolgt (Kästner, 1991). Allerdings sind Boden und Grundwasser, die oft mit chlorierten Ethenen verunreinigt sind, inhomogen in Bezug auf ihre Sauerstoffkonzentration und schließen mikrooxische Zonen ein. Für den mikroaeroben OHRB *S. multivorans* konnte in dieser Arbeit gezeigt werden, dass der Organismus PCE in Anwesenheit von Sauerstoffkonzentrationen bis zu 0,19 mg/ml (0,5% in der Gasphase) reduktiv dehalogenieren kann. Diese Fähigkeit ist von entscheidender Bedeutung, da sie die Durchführbarkeit von Bioremediationsmaßnahmen an mikrooxischen, kontaminierten Standorten erleichtern kann.

5. Zusammenfassung

Sulfurospirillum multivorans ist in der Lage, die reduktive Dechlorierung von Tetrachlorethen (PCE) an die Energiegewinnung über Elektronentransportphosphorylierung zu koppeln (Organohalid-Respiration). Das Schlüsselenzym dieser anaeroben Atmung ist die reduktive PCE-Dehalogenase (PceA). Im Rahmen dieser Arbeit wurde untersucht, welche Proteine und mögliche andere Komponenten (z.B. Chinone) an der Elektronenübertragung zwischen den Enzymen - verantwortlich für die Oxidation der Elektronendonoren - und PceA beteiligt sind.

Im Rahmen einer vergleichenden Genomanalyse mit den Genomen nicht-dechlorierender *Sulfursospirillum*-Arten konnten im Genom von *S. multivorans* Gene identifiziert werden, die für Proteine kodieren, die direkt (z.B. putative Chinol-Dehydrogenase) oder indirekt (z.B. *de novo* Corrinoid-Biosynthese) an der Organohalid-Atmung beteiligt sind. Diese Gene sind alle in der als OHR-(Organohalid-Respiration) Region bezeichneten Genomregion lokalisiert, in der auch das *pceA* Gen kodiert ist. Anschließend konnte in einer vergleichenden Proteomanalyse die PCE-abhängige Regulation dieser Gene beobachtet werden. Wurde *S. multivorans* lange Zeit in Abwesenheit von PCE kultiviert, konnten Proteine, kodiert in der OHR-Region, nicht mehr in den Zellen von *S. multivorans* detektiert werden. Durch erneute Kultivierung in Anwesenheit von PCE konnte die Produktion dieser Proteine wieder induziert werden. Diese Art der Regulation stützt die Annahme, dass die in dieser Region lokalisierten Gene für Proteine kodieren, die entweder direkt oder indirekt an der PCE-Atmung von *S. multivorans* beteiligt sind. Als wahrscheinlichster Kandidat für die Regulation der Organohalid-Atmung wurde der Response-Regulator SMUL_1539 identifiziert.

Eine putative NapGH-ähnliche Chinol-Dehydrogenase, bestehend aus einer membranintegralen Komponente (SMUL_1542) sowie einer periplasmatisch lokalisierten Komponente (SMUL_1541), wurde als wahrscheinliche an der Elektronenübertragung innerhalb der PCE-Atmungskette von *S. multivorans* beteiligte Komponente identifiziert. Anschließend wurde die periplasmatische, Eisen-Schwefel-Cluster-enthaltende Komponente SMUL_1541 heterolog in *Escherichia coli* exprimiert und gereinigt. Nach Rekonstitution der Eisen-Schwefel-Cluster enthielt das gereinigte Protein 8 mol Eisen pro Mol Enzym, was 50% des vorhergesagten Eisengehalts ausmachte. Das gereinigte Protein SMUL_1541 steht für zukünftige Versuche zur Elektronenübertragung innerhalb der PCE-Atmungskette zur Verfügung.

Die Beteiligung von Menachinon und einem revertierten Elektronentransport an der PCE-Atmung von *S. multivroans* wurde durch Inhibitionsversuche mit dem Menachinon-Analogon 2n-Heptyl-4-hydroxychinolin-N-oxid (HQNO) und Protonophoren wie Carbonylcyanid-p-

trifluormethoxyphenylhydrazon (FCCP) bestätigt. Sowohl durch HQNO als auch durch FCCP konnte die PCE-Atmung in Zellsuspensionen von *S. multivorans* gehemmt werden.
Die Ergebnisse der verschiedenen Versuche zusammenführend lassen sich für die PCE-Atmungskette von *S. multivorans* folgende Aussagen treffen: Die PCE-Atmungskette ist Menachinon-abhängig und die putative Chinol-Dehydrogenase SMUL_1541/SMUL_1542 wahrscheinlich an der Elektronenübertragung innerhalb der Atmungskette beteiligt. Ein revertierter Elektronentransport wird benötigt um die PCE-Atmung in *S. multivorans* anzutreiben.

Die mikrobielle Dechlorierung von PCE war bislang nur unter anaeroben Bedingungen beschrieben. In einer anschließenden Studie wurde die Fähigkeit des mikroaeroben Organismus *S. multivorans*, PCE in Anwesenheit von Sauerstoffkonzentrationen unter 0,5% dechlorieren zu können, gezeigt. Die Ergebnisse dieser Studie beinhalten wichtige Parameter für weiterführende Untersuchungen zur reduktiven Dehalogenierung in oxisch-anoxischen Zonen von PCE-kontaminierten Standorten.

6. Summary

Sulfurospirillum multivorans is able to couple the reductive dechlorination of tetrachloroethene (PCE) to energy conservation via electron transport phosphorylation (organohalide respiration). The key enzyme of this anaerobic respiration is the reductive PCE dehalogenase (PceA). In this thesis, we investigated which proteins and possible other components (for example, quinones) are involved in the electron transfer between the enzymes, responsible for the oxidation of the electron donors, and PceA.

By comparing the genome of *S. multivorans* with the genomes of non-dechlorinating *Sulfursospirillum* species, genes encoding proteins involved directly (e.g., putative quinol dehydrogenase) or indirectly (e.g., *de novo* corrinoid biosynthesis) in organohalide respiration have been identified. These genes are all located in the genome region designated as the OHR (organohalide respiration) region, in which the *pceA* gene is also encoded. Subsequently, the PCE-dependent regulation of these genes was shown in a comparative proteome analysis. When *S. multivorans* was cultivated for a long time in the absence of PCE, proteins, encoded in the OHR region, could no longer be detected in the cells of *S. multivorans*. Protein production could be induced again by cultivating *S. multivorans* in the presence of PCE. This type of regulation supports the assumption that the genes located in this region encode proteins that are involved either directly or indirectly in the PCE respiration of *S. multivorans*. The response regulator SMUL_1539 was identified as the most likely candidate for the regulation of organohalide respiration.

A putative NapGH-like quinol dehydrogenase consisting of a membrane integral component (SMUL_1542) and a periplasmatic component (SMUL_1541) was identified as a probable component involved in electron transfer within the PCE respiratory chain of *S. multivorans*. Subsequently, the periplasmic, iron-sulfur cluster-containing component SMUL_1541 was heterologously expressed in *Escherichia coli* and purified. After reconstitution of the iron-sulfur clusters, the purified protein contained 8 moles of iron per mole of enzyme, what representes 50% of the predicted iron content. The purified protein SMUL_1541 is available for future experiments on electron transfer within the PCE respiratory chain.

The involvement of menaquinone and a reverse electron transport in PCE respiration of *S. multivorans* was investigated and confirmed by inhibition experiments with the menaquinone analog 2-n-heptyl-4-hydroxychinolin N-oxide (HQNO) and protonophores such as Carbonyl cyanide-p-(trifluoromethoxy)-phenylhydrazone (FCCP). PCE respiration was inhibited in cell suspensions of *S. multivorans* by using HQNO as well as by using FCCP.

The results of the various experiments summarizing the following statements can be made for the PCE respiratory chain of *S. multivorans*: The PCE respiratory chain is menaquinone-dependent and the putative quinol dehydrogenase SMUL_1541 / SMUL_1542 is probably involved in the electron transfer within the respiratory chain. The reverse electron flow is required to drive the PCE respiration.

Until now, the microbial dechlorination of PCE has only be described under anaerobic conditions. In this study, the ability of the microaerobic organism *S. multivorans* to dechlorinate PCE in the presence of oxygen concentrations below 0.5% was demonstrated. The findings of this study are important in studies on reductive dehalogenation in oxic-anoxic zones of PCE-contaminated sites.

7. Literatur

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Danksagung

An dieser Stelle möchte ich mich bei allen Personen bedanken, die mir auf meinem Weg zur Dissertation tatkräftig zur Seite standen.

Frau Prof. Dr. Gabriele Diekert möchte ich für die Bereitstellung des interessanten Themas meiner Dissertation und die stete Diskussionsbereitschaft danken.

Für die engagierte Betreuung meiner Doktorarbeit, die fachlichen Diskussionen sowie die vielen hilfreichen Ratschläge und Anregungen gilt mein besonderer Dank Herrn Dr. Torsten Schubert und Herrn Dr. Tobias Goris.

Für die hervorragende und unkomplizierte Zusammenarbeit zur Durchführung der Proteomanalysen möchte ich mich ganz besonders bei Christian Schiffmann und Herrn Dr. Nico Jehmlich bedanken (Umweltforschungszentrum Leipzig).

Mein besonderer Dank gilt zudem allen Mitarbeitern des Instituts fur Angewandte und Ökologische Mikrobiologie für die gute Arbeitsatmosphäre und für hilfreiche, fachliche Diskussionen sowie viele praktische Tipps.

Ganz besonderer Dank gilt außerdem meinen Eltern und Geschwistern für ihre in jeder Beziehung und zu jeder Zeit perfekte Unterstützung.

Ehrenwörtliche Erklärung

Hiermit bestätige ich, Jennifer Gadkari, geboren am 17.03.1983 in Bayreuth, dass mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist. Ich habe die vorliegende Dissertation selbstständig angefertigt außer den angegebenen keine Hilfsmittel, persönlichen Mitteilungen oder Quellen verwendet. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen. Es wurden von mir keine geldwerten Leistungen erbracht, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Diese Dissertation wurde nur dem Fakultätsrat der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena und keiner anderen Hochschule zur wissenschaftliche Prüfung oder Dissertation vorgelegt. Diese Arbeit ist weder identisch noch teilidentisch mit einer Arbeit, welche an der Friedrich-Schiller-Universität Jena oder einer anderen Hochschule zur Dissertation eingereicht worden ist.

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Wissenschaftliche Veröffentlichungen

Tobias Goris, Torsten Schubert, Jennifer Gadkari, Tesfaye Wubet, Mika Tarkka, Francois Buscot, Lorenz Adrian and Gabriele Diekert (2014) Insights into organohalide respiration and the versatile catabolism of *Sulfurospirillum multivorans* gained from comparative genomics and physiological studies. Environ Microbiol **16**: 3562–3580.

Tobias Goris*, Christian L. Schiffmann*, Jennifer Gadkari*, Torsten Schubert, Jana Seifert, Nico Jehmlich, Martin von Bergen & Gabriele Diekert (2015) Proteomics of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates. Sci. Rep. 5, 13794; doi: 10.1038/srep13794.
(* geteilte Erstautorenschaft)

Tobias Goris* , Christian L. Schiffmann*, Jennifer Gadkari*, Lorenz Adrian, Martin von Bergen, Gabriele Diekert, Nico Jehmlich (2016) Proteomic data set of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates. Data Brief **8**: 637-642.
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Jennifer Gadkari*, Tobias Goris*, Christian L. Schiffmann*, Raffael Rubick, Lorenz Adrian, Torsten Schubert, Gabriele Diekert. Reductive tetrachloroethene dehalogenation in the presence of oxygen by *Sulfurospirillum multivorans*: Physiological studies and proteome analysis.
(* geteilte Erstautorenschaft)

- eingereicht bei FEMS Microbiology Ecology am 23.06.2017

Vorträge im Rahmen von wissenschaftlichen Tagungen

2015 Identification of tetrachloroethene respiratory chain components in *Sulfurospirillum multivorans*

Jennifer Gadkari, Tobias Goris, Christian Schiffmann, Nico Jehmlich, Sven Baumann, Martin von Bergen, Torsten Schubert and Gabriele Diekert
Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM), Marburg

Poster im Rahmen von wissenschaftlichen Tagungen

2012 Elucidation of the tetrachloroethene respiratory chain in *Sulfurospirillum multivorans*

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Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM), Tübingen

2013 Identification and characterization of tetrachloroethene respiratory chain components in *Sulfurospirillum multivorans*

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